AD_____

Award Number: W81XWH-04-1-0222

TITLE: Analysis of Morphogenic Effect of hDAB2IP on Prostate Cancer and its Disease Correlation

PRINCIPAL INVESTIGATOR: Jer-Tsong Hsieh, Ph.D.

CONTRACTING ORGANIZATION: Texas Southwestern Medical Center University
Dallas, Texas 75390-9105

REPORT DATE: February 2005

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	February 2005	Annual (1 Feb 2004 - 31 Jan 2005)

4. TITLE AND SUBTITLE
Analysis of Morphogenic Effect of hDAB2IP on Prostate Cancer and its Disease Correlation

5. FUNDING NUMBERS
W81XWH-04-1-0222

6. AUTHOR(S)
Jer-Tsong Hsieh, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Texas Southwestern Medical Center University
Dallas, Texas 75390-9105

E-Mail: JT.hsieh@UTsouthwestern.edu

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 Words)

It is known that prostate cancer (PCa) acquires its malignant phenotypes by losing the ability to differentiate into mature cells. Therefore, restoration of the normal differentiated phenotype in AIPCa certainly can suppress the progression of PCa, which offers a new therapeutic strategy for PCa treatment. Using molecular biologic approaches, our laboratory was able to unveil an altered genetic make-up that is involved in cell differentiation in normal prostatic epithelium. Data from our laboratory has documented the potential role of this novel gene, hDAB2IP, in the cell differentiation of normal prostatic epithelia. In PCa, hDAB2IP is often down regulated. Thus, we proposed to study the underlying mechanism leading to the loss of hDAB2IP gene expression. In addition to the genetic mutations of tumor suppressor genes associated with PCa, recent data clearly indicate that epigenetic alterations are also involved in this silencing. In this study, we have shown that both histone methylation and histone deacetylation play an important in silencing hDAB2IP gene expression and this event is mediated by a poplycomb group transcription complex Ezh2. The outcome of this study provides a better understanding of the fundamental changes between normal prostatic basal/stem cell and AIPCa, and further lead to the development of a novel and more effective intervention for this disease.

14. SUBJECT TERMS
Cell differentiation, GTPase activating protein, tumor suppressor, epigenetic regulation, signal transduction, prostate cancer

15. NUMBER OF PAGES
99

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

Table of Contents

INTRODUCTION

It has been well documented that alterations in genomic DNA, such as point mutations, homozygous deletions, and loss of heterozygosity are linked to the pathogenesis of cancer (1), including PCa (2). However, the majority of studies have focused on the DNA sequence and to a lesser extent on DNA structure and its surrounding environment. Recently, investigators started looking at epigenetics as an alternative and complementary mechanism in the pathogenesis of cancer. The term "epigenetic" refers to the heritable changes in gene expression that are caused by mechanisms other than the alteration in the nucleotide sequence (3). This concept generated tremendous new knowledge in understanding gene expression in mammalian cells.

Human DAB2IP (hDAB2IP), a novel GTPase-activating protein modulating the Ras-mediated signaling and TNF-mediated apoptosis, is a potent growth inhibitor in human prostate cancer (PCa). Loss of *hDAB2IP* expression in PCa is due to altered epigenetic regulation (i.e., DNA methylation and histone modification) of its promoter region. The elevated polycomb Ezh2, a histone methyltransferase (HMTase), has been associated with PCa progression. In this study, we have studied the potential role of Ezh2 expression in modulating hDAB2IP expression in PCa and its mechanism of action in histone modification. The information generated from this study provides a better understanding of the functional role of Ezh2 in metastatic PCa and the mechanism of hDAB2IP gene silencing, which will help not only early diagnosis of AIPCa and also development of new intervention for PCa.

RECENT PROGRESS

In the first year, two manuscripts and one review manuscript have been submitted or accepted for publication (Appendix 1-3). Overall, Task 1 is 75% completed; Task 2 will begin shortly. Detailed progress of these aims is outlined below.

Task 1 To delineate mechanism(s) leading to the down regulation of hDAB2IP gene in PCa and correlate the status of the hDAB2IP in PCa specimens with disease progression.

A. <u>The role of histone modification in modulating hDAB2IP gene expression in prostatic epithelia and PC cells.</u>

Recent data indicate that the elevated Ezh2 levels are found in hormone-refractory, metastatic PCa (4, 5) as well as in poorly differentiated breast carcinomas (6, 7). Human Enhancer of Zeste homolog (Ezh2) protein belongs to Polycomb repressive complex 2 (8) that also includes Eed, Suz12 and the histone-binding protein RbAp48/46 (9-13). The Ezh2 complex appears to be a transcription repressor that has been shown in cellular memory system, X-inactivation, germline development, stem cell pluripotency and cancer metastasis (14-19). This complex exhibits an intrinsic histone lysine methyltransferase (HKMT) activity on histone H3 lysine (K) 27 and 9 or histone H1 K26 medicated by the SET domain of Ezh2 (8, 10, 11, 20, 21). However, the underlying mechanism of Ezh2 in these cancer cells is still unknown.

We found an inverse correlation between Ezh2 and hDAB2I*P* gene expression between normal prostatic epithelia and PCa cells (see Fig. 1 in Appendix 1). The knockdown of Ezh2

expression by siRNA in the PCa cells resulted in an elevated hDAB2I*P* gene expression (see Fig. 2 in Appendix 1). In contrast, Increased Ezh2 expression in normal prostatic epithelial cells could inhibit the hDAB2IP promoter activity and its gene expression (see Fig. 3 in Appendix 1).

To demonstrate the direct interaction of Ezh2 complex with hDAB2IP promoter region, the chromosome immunoprecipitation (ChIP) assay was employed. A robust binding of Ezh2 as well as two other components Eed and Suz12 to the hDAB2IP promoter was seen in PC3 cells, the moderate binding of Ezh2 complex was detected in DU145 cells and the lowest binding of Ezh2 complex was detected in PZ-HPV-7 cells. A higher levels of histone deacetylase 1 (HDAC1) associated with hDAB2IP promoter were detected in PC3 and DU145 cells more than in PZ-HPV-7 cells. In contrast, a lower level of acetyl H3 associated with *hDAB2IP* promoter was seen in these two PCa cells than in PZ-HPV-7 cells. These data indicate that this repression complex associated with *hDAB2IP* promoter contains both Ezh2 complex and HDAC1 in PCa cells (see Fig. 4 in Appendix 1).

Furthermore, by increasing Ezh2 gene expression in PZ-HPV-7 cells, we observed that the presence of Ezh2 protein could recruit not only other PcG proteins such as Eed and Suz12 but also HDAC1 to the hDAB2IP promoter region in these cells. Meanwhile, the decreased acetyl H3 levels became more apparent, suggesting that Ezh2 plays a key role in recruiting other transcription repressors to the hDAB2IP promoter region in prostatic epithelia (see Fig. 5 in Appendix 1).

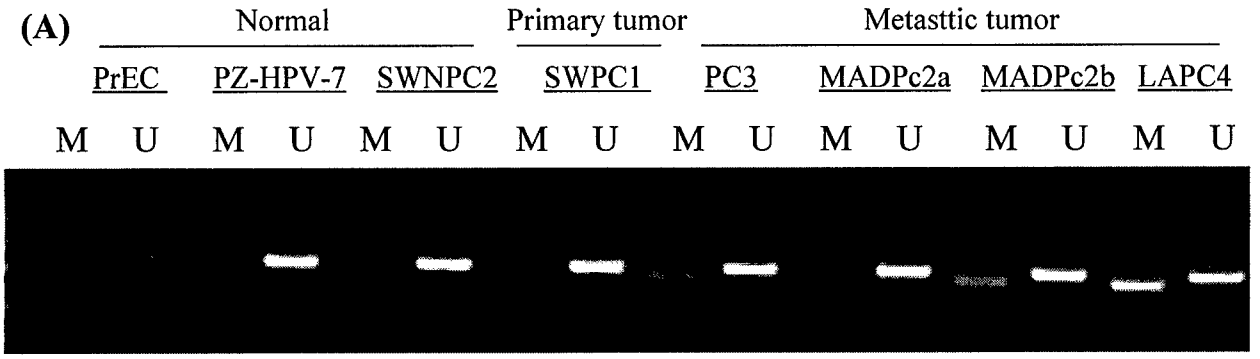
In this study, our data indicate that the levels of Ezh2 associated with hDAB2IP promoter region are consistent with the steady-state levels of hDAB2I*P* mRNA and protein in all three cell lines tested. Most importantly, Ezh2 plays an active role in recruiting other PcG components such as Eed and Suz12 to form a functional complex along with HDAC1 to the promoter region of hDAB2IP gene. Based on these results, one could predict that the presence of elevated Ezh2 levels in PCa signifies the down regulation of tumor suppressor genes via the cooperative effect of histone methylation and deacetylation. This study provides further evidence to support the critical role of epigenetic gene regulation in PCa progression.

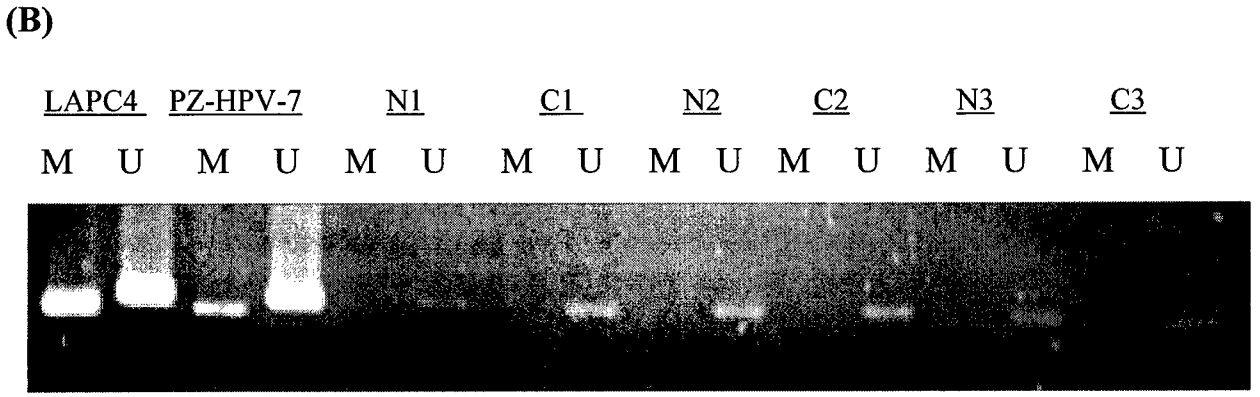
B. <u>The status of DNA hypermethylation associated with hDAB2IP gene promoter region in PCa cell lines and tissue specimens.</u>

Recently, we reported that the P2 region (-598 to +44) in the hDAB2IP gene showed promoter activity and that the transcriptional silencing by the aberrant methylation of the P2 region was a critical event during the tumorigenesis of PCa (22). In order to determine the DNA methylation status of hDAB2IP gene promoter in clinical specimens, methylation-specific PCR (MSP) assay, one of the convenient and sensitive assays to examine the methylation status developed by Herman (23), was employed. The primer is one of the most essential elements for MSP assay and ideally should be designed based on bisulfited DNA sequence and expression in promoter region; we have designed MSP primers in promoter region and found the methylation status of hDAB2IP gene in several PCa cell line and normal prostatic epithelia to be consistent with our previous report (22). Subsequently, we performed MSP assay using PCa specimens isolated from organ-confined patients (20 specimens in total, ranging from Gleason's grade from 6 to 9) and found that the hDAB2IP gene promoter is not hypermethylated. This result may have several implications: (1) DNA methylation may occur in other regions from P2 region of

hDAB2IP gene promoter; (2) DNA methylation may only occur in metastatic PCa based on *in vitro* data; (3) DNA methylation of hDAB2IP gene promoter may not associate with PCa specimens. Currently, we are examining other region in DAB2IP gene promoter and also we are collecting specimens derived from metastatic PCa patients.

Fig. 1 Determination the status of DNA methylation in hDAB2IP gene promoter region in PCa cell lines and tissue specimens. High molecular weight DNA isolated from various prostate cell lines (A) and tissue specimens (B) was treated with sodium bisulfite and subjected to MSP assay. M: methylated primer; U: unmethylated primer; N: normal; C: cancer.





C. Cloning and characterizing DAB2IP gene and its promoter from mouse.

We are planning to generate DAB21P knock out mouse. Thus, we have cloned mouse DAB2IP gene and characterized its promoter (see Appendix 2). Overall, the *mDAB2IP* gene contains 14 exons and 13 introns and spans approximately 65kb. Exon1 contains at least three variants, exon Ia; exon Ib and exon Ic. The deduced amino acid sequence of mouse DAB2IP encompasses 1065 residues containing several unique protein interaction motifs as well as a Ras-like GAP-related domain, which shares a high homology among human and rat. We have mapped an 800 bp segment containing a 5'-upstream sequence from exon Ia as a promoter region (–147/+545) in prostatic epithelial cell lines (TRAMP-C); this region is highly GC-rich and *mDAB2IP* appears to be a TATA-less promoter.

KEY RESEARCH ACCOMPLISHMENT

- Characterize the role of histone methylation in modulating hDAB2IP gene expression in PCa cells.
- Characterize the elevated Ezh2 levels correlated with down regulation of hDAB2IP gene expression in PCa.
- Delineate the role of Ezh2 in suppressing hDAB2IP gene expression in normal prostatic epithelia.
- Identify the recruitment of histone deacetylase by Ezh2 complex to hDAB2IP gene promoter region in normal prostatic epithelia.
- Define the specific methylation site in H3 associated with hDAB2IP promoter region catalyzed by Ezh2 in normal prostatic epithelia.
- Characterize the DNA methylation status of hDAB2IP gene promoter in PCa cell lines and specimens.
- Cloning and characterization of mouse DAB2IP gene and its promoter.

REPORTABLE OUTCOMES

FULL-LENGTH PAPER

1. Chen, H, and Hsieh, J.T. (2005) Down regulation of human DAB2IP gene expression mediated by polycomb Ezh2 complex and histone deacetylase in prostate cancer. J. Biol. Chem. (submitted).
2. Chen, H, Karam. J.A., Schultz, R., Zhang, Z., Ducan, C., and Hsieh, J.T. (2005) Cloning of *mDAB2IP* gene, a novel member of the RasGTPase-activating protein family and characterization of its regulatory region. Cytogenetics and Genome Res., (submitted)

REVIEW PAPER

1. Karam J., Benaim, E., Chen, H., Pong, R.C., and Hsieh, J.T. (2005) Epigenetics in prostate cancer. In Prostate Cancer: Mechanisms, Prevention, and Hormone Therapy (Chang, C., ed) (In press)

CONCLUSIONS

In addition to the genetic mutations of tumor suppressor genes associated with PCa, recent data clearly indicate that epigenetic alterations are also involved in this silencing. Unlike genetic changes, epigenetic modifications are potentially reversible, which can open a new

avenue of cancer therapy. In this project, we unveiled the mechanism action of Ezh2, a HMTase often elevated in metastatic PCa, in suppressing hDAB2IP gene expression. This is the first report to detail the underlying mechanism of Ezh2 in PCa. Also, we also unveiled that a HMTase can recruit HDAC to form a transcription repression complex. It is also interesting to examine whether DNA methyltransferase can be associated with this complex in hDAB2IP gene promoter region. Nevertheless, these data clearly indicate that the role of epigenetic control of tumor suppressor gene during PCa progression. To validate all these *in vitro* data, we are currently establishing tissue ChIP assay to examine clinical specimens.

REFERENCES

1. Knudson, A.G. (1985) Hereditary cancer, oncogenes, and antioncogenes. Cancer Res 45,1437-1443
2. Ozen, M, Pathak, S. (2000) Genetic alterations in human prostate cancer: a review of current literature. Anticancer Res. 20,1905-1912
3. Bird, A. (2002) DNA methylation patterns and epigenetic memory. Genes Dev, 16,6-21
4. Varambally, S. *et al.* (2002) The polycomb group protein EZH2 is involved in progression of prostate cancer. Nature, 419, 624-629
5. Rhodes, D.R. *et al.* (2003) Multiplex biomarker approach for determining risk of prostate-specific antigen-defined recurrence of prostate cancer. J. Natl. Cancer Inst. 95, 661-668
6. Kleer, C.G. *et al.* (2003) EZH2 is a marker of aggressive breast cancer and promotes neoplastic transformation of breast epithelial cells. Proc. Natl. Acad. Sci. USA 100, 11606-11611
7. Raaphorst, F.M. *et al.* (2003) Poorly differentiated breast carcinoma is associated with increased expression of the human polycomb group EZH2 gene. Neoplasia. 5, 481-488
8. Kuzmichev, A., Jenuwein, T., Tempst, P., Reinberg, D. (2004) Different EZH2-containing complexes target methylation of histone H1 or nucleosomal histone H3. Mol. Cell. 14, 183-193.
9. Cao, R., Zhang, Y. (2004) The functions of E(Z)/EZH2-mediated methylation of lysine 27 in histone H3. Curr. Opin. Genet. Dev. 14, 155-164
10. Kuzmichev, A. *et al.* (2002) Histone methyltransferase activity associated with a human multiprotein complex containing the Enhancer of Zeste protein. Genes. Dev. 16, 2893-2905
11. Cao, R. *et al.* (2002) Role of histone H3 lysine 27 methylation in Polycomb-group silencing. Science 298, 1039-1043
12. Pasini, D. *et al.* (2004) Suz12 is essential for mouse development and for EZH2 histone methyltransferase activity. EMBO. J. 23, 4061-4071
13. Cao, R., Zhang, Y. (2004) SUZ12 is required for both the histone methyltransferase activity and the silencing function of the EED-EZH2 complex. Mol. Cell. 15, 57-67
14. Jacobs, J.J., van Lohuizen, M. (2002) Polycomb repression: from cellular memory to cellular proliferation and cancer. Biochim. Biophys. Acta. 1602, 151-161

15. O'Carroll, D. *et al.* (2001) The polycomb-group gene Ezh2 is required for early mouse development. Mol. Cell. Biol. 21, 4330-4336
16. Plath, K. *et al.* (2003) Role of histone H3 lysine 27 methylation in X inactivation. Science 300, 131-135
17. Silva, J. *et al.* (2003) Establishment of histone H3 methylation on the inactive X chromosome requires transient recruitment of Eed-Enx1 polycomb group complexes. Dev. Cell. 4, 481-495
18. Kelly, W. G. *et al.* (2002) X-chromosome silencing in the germline of C. elegans. Development 129, 479-492
19. Fong, Y. *et al.* (2002) Regulation of the different chromatin states of autosomes and X chromosomes in the germ line of C. elegans. Science 296, 2235-2238
20. Czermin, B. *et al.* (2002) Drosophila enhancer of Zeste/ESC complexes have a histone H3 methyltransferase activity that marks chromosomal Polycomb sites. Cell 111, 185-196.
21. Muller, J. *et al.* (2002) Histone methyltransferase activity of a Drosophila Polycomb group repressor complex. Cell 111, 197-208
22. Chen, H. *et al.* (2002) Epigenetic regulation of a novel tumor suppressor gene (hDAB2IP) in prostate cancer cell lines. J. Biol. Chem., 278, 3121-3130.
23. Herman, J.G. *et al.* (1996). Methylation-specific PCR: a novel PCR assay for methylation status of CpG islands. Proc. Natl. Acad. Sci. USA, 93, 9821-9826.

DOWN REGULATION OF HUMAN DAB2IP GENE EXPRESSION MEDIATED BY POLYCOMB EZH2 COMPLEX AND HISTONE DEACETYLASE IN PROSTATE CANCER

Hong Chen and Jer-Tsong Hsieh*

Department of Urology, University of Texas Southwestern Medical Center, Dallas, TX 75390-9110

Running title: Ezh2 regulation of *hDAB2IP* gene expression

* Address all correspondence to: J.T. Hsieh, Ph.D., University of Texas Southwestern Medical Center, Department of Urology, 5323 Harry Hines Blvd., Dallas, TX 75390-9110, (phone) 214-648-3988, (fax) 214-648-8786, (e-mail) JT.Hsieh@UTSouthwestern.edu

SUMMARY

Human DAB2IP (hDAB2IP), a novel GTPase-activating protein modulating the Ras-mediated signaling and TNF-mediated apoptosis, is a potent growth inhibitor in human prostate cancer (PCa). Loss of *hDAB2IP* expression in PCa is due to altered epigenetic regulation (i.e., DNA methylation and histone modification) of its promoter region. The elevated polycomb Ezh2, a histone methyltransferase (HMTase), has been associated with PCa progression. In this study, we demonstrated that increased Ezh2 expression in normal prostatic epithelial cells could inhibit *hDAB2IP* gene expression, in contrast, reduced endogenous Ezh2 expression in PCa by specific siRNA could lead to increased expression of hDAB2IP. Furthermore, the association of Ezh2 complex (including Eed and Suz12) with *hDAB2IP* gene promoter was highly detectable in PCa cells but not in normal prostatic epithelial cells. Increased Ezh2 expression in normal prostatic epithelial cells by cDNA transfection facilitated the recruitment of other component of Ezh2 complex to the *hDAB2IP* promoter region accompanied with increased levels of methyl histone 3 (H3) and histone deacetylase (HDAC1). We further unveiled that the methylation status of lysine (K) 27 but not K9 of H3 in *hDAB2IP* promoter region correlated with the *hDAB2IP* levels in both normal prostatic epithelial cells and PCa cells. Together, we conclude that the altered epigenetic regulation of *hDAB2IP* gene in PCa can be due to the elevated levels of Ezh2, which provides an underlying mechanism of the functional role of Ezh2 in metastatic PCa.

INTRODUCTION

The human DOC-2/DAB2 interactive protein gene (*hDAB2IP*) located at chromosome 9q33.1-33.3 is a new member of the Ras GTPase-activating family gene (1, 2). Our recent data indicate that hDAB2IP protein is a growth inhibitor in prostate cancer (PCa) cells (3). In addition, hDAB2IP protein

(also named ASK-interacting protein 1 [AIP1]) is involved in TNF-mediated JNK signaling pathway that leads to cell apoptosis (4, 5). We have demonstrated that normal prostatic epithelial cells express higher hDAB2IP levels than PCa cells, which is due to epigenetic alternation (i.e. aberrant DNA methylation and histone deacetylation) in the promoter region during carcinogenesis. Similarly, loss of *hDAB2IP* expression was also detected in breast and lung cancer specimens (6, 7) frequently associated with the promoter hypermethylation.

Human Enhancer of Zeste homolog (Ezh2) protein belongs to Polycomb repressive complex 2 (8) that also includes Eed, Suz12 and the histone-binding protein RbAp48/46 (9-13). The Ezh2 complex appears to be a transcription repressor that has been shown in cellular memory system, X-inactivation, germline development, stem cell pluripotency and cancer metastasis (14-20). This complex exhibits an intrinsic histone lysine methyltransferase (HKMT) activity on histone H3 lysine (K) 27 and 9 or histone H1 K26 medicated by the SET domain of Ezh2 (8, 10, 11, 21, 22).

Recent data indicate that the elevated Ezh2 levels are found in hormone-refractory, metastatic PCa (23, 24) as well as in poorly differentiated breast carcinomas (25, 26). However, the underlying mechanism of Ezh2 in these cancer cells is still unknown. In this study, we found an inverse correlation between *Ezh2* and *hDAB2IP* gene expression between normal prostatic epithelia and PCa cells. Increased Ezh2 expression in normal prostatic epithelial cells could inhibit the *hDAB2IP* promoter activity and its gene expression. In contrast, the knockdown of *Ezh2* expression by siRNA in the PCa cells resulted in an elevated *hDAB2IP* gene expression. These data prompted us to investigate the role of Ezh2 in modulating *hDAB2IP* gene expression and we demonstrated that Ezh2 complex and histone deacetylase (HDAC) are associated with *hDAB2IP* promoter regions in PCa cells, but not in normal prostatic epithelial cells. The outcome of this study provides an underlying mechanism of the functional role of Ezh2 in metastatic PCa.

EXPERIMENTAL PROCEDURES

Cell Cultures

Three human prostate cancer cell lines (LNCaP, C4-2, and PC3) were maintained in T medium supplemented with 5% fetal bovine serum (27). MDAPCa 2a and MDAPCa 2b cell lines derived from patients with bony metastasis (28) were maintained in BRFF-HPC1 medium (Biological Research Faculty and Facility, Inc., Jamsville, MD) supplemented with 20% fetal bovine serum. VCAP cell line derived from a vertebral metastatic lesion of prostate cancer (29) and DU145 were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum. Three normal human prostate epithelial cells (PrEC1, PrEC2 and PrEC3) were maintained in chemical-defined medium (PrEGM) purchased from Biowhitaker. PZ-HPV-7 (an immortalized cell line derived from the peripheral zone of a normal prostate) (30, 31), and three additional primary prostatic epithelial cells (SWPC1, SWPC2 and SWPC3), derived from cancer lesions, and SWNPC2 derived from the adjacent normal tissue were maintained in the PrEGM medium.

Transient Transfection and luciferase reporter gene assay

Cells were plated at a density of 1.8×10^5 cells in per 6-well plate. After 24 hrs, PrEC1 and PZ-HPV-7 cells were co-transfected with myc-tagged human Ezh2 expression construct (32) and a luciferase reporter vector containing the *hDAB2IP* promoter-pGL3-P2 (2) using Lipofectamine Plus transfection reagent (Invitrogen). Luciferase assay was described previously (1, 2). All experiments were repeated at least three times in triplicate. The relative Luciferase activity (RLA) was calculated as described previously (2).

RNA isolation and quantitative RT-PCR (qRT-PCR)

Total cellular RNA was isolated using RNeasy Kit (QIAGEN, Inc) according to the manufacturer's instruction. To measure *hDAB2IP* or *Ezh2* mRNA levels, 1 μg of total cellular RNA from each cell line was transcribed into first strand cDNA using iScript™ cDNA Synthesis Kit (BIO-RAD, Inc.).

The first strand cDNA was further amplified by qRT-PCR using either hDAB2IP primer set F-hDAB2IP, 5'-TGGACGATGTGCTCTATGCC-3'; R-hDAB2IP, 5'-GGATGGTGATGGTTTGGTAG-3' or Ezh2 primer set F-Ezh2, 5'-GCCAGACTGGGAAGAAATCTG-3'; R-Ezh2, 5'-TGTGCTGGAAAATCCAAGTCA-3' in a 40-μl reaction mixture containing 20-μl iQ™ SYBGREEN Supermix® (BIO-RAD, Inc). The reactions were carried out in a 96-well plate and PCR amplification protocol was followed by 95°C (3 min) and 36 cycles of amplification cycle (95°C [30 sec], 55°C [30 sec], and 72°C [1 min]) using in iCycler iQ machine (BIO-RAD, Inc.). The 18S cDNA (F-18S, 5'-GGAATTGACGGAAGGGCACCACC-3'; R-18S, 5'-GTGCAGCCCCGGACATCTAAGG –3';) was used as an internal control. All experiments have been repeated twice in duplicates. *hDAB2IP* or *Ezh2* mRNA level was determined by normalizing with the 18S cDNA of each sample.

The quality control was carried out using both electrophoresis analyses on a 2% NuSieve agarose gel (3:1, FMC Bioproducts) and melting curve analysis performed immediately after the end of amplification using 95°C (1 min), 55°C (1 min) and 80 cycles of 0.5°C increment beginning at 55°C. We also performed the standard curve for hDAB2IP and Ezh2 to ensure the linearity and efficiency of both genes.

Western blot analysis

Cells were lysed with whole-cell lysis buffer (50mM HEPES, 150mM NaCl, 1.5 mM $MgCl_2$, 0.5mM EDTA, 10% glycerol, 1% Triton X-100, 10mM NaF, 1mM DTT, 1mM PMSF) and were alternately frozen and thawed three times in –80°C to rupture the cell membranes. The samples were incubated for 30 min on ice to lyse the nuclei and then centrifuged at 4000Xg for 5 min to pellet the cell membranes. The protein concentration of each sample was determined by a standard Bradford assay. Equal amounts of protein (20μg) of each cell line were subjected to western blot analysis. Antibodies used for probing were hDAB2IP (3), Lamin A/C (Upstate Inc), Ezh2 (Upstate Inc), and Actin (SIGMA Co).

RNA interference

Two different 21-nt duplex siRNAs for Ezh2, (5'-AAGAGGTTCAGACGAGCTGAT-3'

(23), 5'-AAGACTCTGAATGCAGTTGCT-3' (33), or control siRNA for Lamin A/C (5'-CTG GACTTCCAGAAGAACA-3' were synthesized by QIAGEN. Twenty-four hours after plating, the cells were transfected either with both Ezh2 siRNA duplexes together (100 nmoles each) or with control siRNA (200 nmoles) using RNAiFect Transfection Reagent (QIAGEN) according to the manufacturers' instructions. At various time points after transfection, the cells were harvested and subjected to western blot analysis.

Chromatin Immunoprecipetion (ChIP) assay

This assay was performed as described previously (2). Briefly, precleared chromatin from 2 x10^6 cells was used for each ChIP assay and 5μg of each antibody used in this assay including Ezh2; Suz12, trimethyl H3 (K27), trimethyl H3 (K9), and dimethyl H3 (K-9) purchased from Abcam Inc., Eed, dimethyl H3 (K27), monomethyl H3 (K27), acetyl H3, and HDAC1 purchased from Upstate. The total input or immunoprecipitated DNA was determined by qPCR at least twice. ΔCt (threshold cycle) of each sample = mean of $Ct_{(antibody)}$ – mean of $Ct_{(input)}$.

RESULTS

Profiling *hDAB2IP* and *Ezh2* expression in various prostatic epithelia

To evaluate the expression profile of *hDAB2IP* gene and *Ezh2* in the prostate cell lines, we performed qRT-PCR to document the steady-state levels of *hDAB2IP* (Figure 1A) and *Ezh2* (Figure 1B) mRNA in cells derived from normal prostatic epithelium (PrEC1, PrEC2, PrEC3, PZ-HPV-7 and SWNPC2), primary PCa cells (SWPC1, SWPC2 and SWPC3), or metastatic PCa cell lines (LNCaP, C4-2, PC3, MADPC2a, MADPC2b, VCAP and DU145). We observed a trend of decreased *hDAB2IP* mRNA from normal cells to malignant cells, however, an opposite trend of *Ezh2* mRNA was seen from normal cells to malignant cells. Western blot data (Figure 1C) also confirmed the qRT-PCR results. Taken together, an inverse correlation between *Ezh2* and *hDAB2IP* gene expression prompted us to investigate the possibility that *hDAB2IP* gene expression is modulated by the Ezh2 complex, particularly, loss of *hDAB2IP* expression has been frequently observed in metastatic PCa cells.

The inhibitory effect of Ezh2 on *hDAB2IP* gene expression

To test if Ezh2 is a negative regulator to *hDAB2IP* gene in PCa cells, western blot analysis was performed using whole-cell extract prepared from PCa cells transfected with either a mixture of two different Ezh2 siRNAs (23, 33) or lamin A/C siRNA. The significant decreased Ezh2 levels in PC3 (Figure 2A) and in DU145 (Figure 2C) were observed within 48 to 72 hrs after transfection and the reduced Ezh2 protein became more prominent 96 hrs after transfection. Under this condition, the elevated levels of hDAB2IP protein were detected in both PCa cells. In contrast, Lamin A/C siRNA duplex did not alter Ezh2 or hDAB2IP levels in both cells (Figure 2B and D).

To determine the inhibitory effect of Ezh2 on *hDAB2IP* gene expression via transcriptional or post-transcriptional regulation, we examined the *hDAB2IP* promoter activity in several normal prostatic epithelial cells

by transfecting with both pGL3-P2 and Ezh2 expression vectors. We observed that an increased Ezh2 protein expression (Figure 3A) in normal prostatic epithelial cells could inhibit the *hDAB2IP* promoter activity (Figure 3B to E) in a dose- and time-dependent manner. Using qRT-PCR, we also observed a similar inhibitory effect of Ezh2 on *hDAB2IP* mRNA levels in these and two other normal epithelial cells such as PrEC2 and PrEC3 (data not shown). These data clearly indicate that the suppression of *hDAB2IP* gene promoter activity is mediated by Ezh2 protein.

The association between Ezh2 complex and HDAC1 in *hDAB2IP* promoter region in PCa cells

To demonstrate the direct interaction of Ezh2 complex with *hDAB2IP* promoter region, the ChIP assay was employed. In Figure 4A, robust binding of Ezh2 as well as two other components Eed and Suz12 to the *hDAB2IP* promoter was seen in PC3 cells, the moderate binding of Ezh2 complex was detected in DU145 cells and the lowest binding of Ezh2 complex was detected in PZ-HPV-7 cells. Noticeably, the amount of Ezh2 complex associated with *hDAB2IP* promoter inversely correlated with *hDAB2IP* mRNA and protein expression pattern in these cells (Figure 1).

It is known that transcriptional repression mediated by human polycomb group (PcG) protein involves histone deacetylation (34). We therefore determined the association of HDAC1 with the same region of *hDAB2IP* promoter. As shown in Figure 4B, higher levels of HDAC1 associated with *hDAB2IP* promoter were detected in PC3 and DU145 cells more than in PZ-HPV-7 cells. In contrast, a lower level of acetyl H3 associated with *hDAB2IP* promoter was seen in these two PCa cells than in PZ-HPV-7 cells (Figure 4B). These data indicate that this repression complex associated with *hDAB2IP* promoter contains both Ezh2 complex and HDAC1 in PCa cells.

Furthermore, by increasing *Ezh2* gene expression in PZ-HPV-7 cells, we observed that the presence of Ezh2 protein could recruit not only other PcG proteins such as Eed and Suz12 (Figure 5A) but also HDAC1 to the *hDAB2IP* promoter region in these cells. Meanwhile, the decreased acetyl H3 levels became more apparent (Figure 5B), suggesting that Ezh2 plays a key role in recruiting other transcription repressors to the *hDAB2IP* promoter region in prostatic epithelia.

The methylation status of K9 and K27 of H3 in *hDAB2IP* promoter region modulated by Ezh2

Although the human Eed-Ezh2 complex and its Drosophilia ESC-E(Z) counterpart have been shown to be HMKTase (9), its substrate specificity still remains unclear. For example, Cao *et al.*, (11) and Muller *et al*, (22) showed that K27 in H3 is the only amino acid methylated by Ezh2, however other groups (10, 21) demonstrated that Ezh2 can also methylate H3-K9. Moreover, the lysine in H3 exists in mono-, di-, and tri-methyl status (35-37) and their impact on gene regulation is still unclear.

For *hDAB2IP* promoter region, di- and tri-methyl H3-K27 were highly detectable in PC3 and DU145 cells but not in PZ-HPV-7 (Figure 4C), which is consistent with the presence of Ezh2 complex in these three cell lines (Figure 4A). We also noticed that the mono-

methyl H3-K27 did not vary among these three cell lines, suggesting that mono-methyl H3-K27 may reflect a basal status of K27. By increasing Ezh2 protein expression in PZ-HPV-7 cells, we demonstrated that the levels of both di- and tri-methyl but not mono-methyl H3-K27 elevated dramatically in a dose–dependent manner (Figure 5C), suggesting that effect of Ezh2 is to convert H3-K27 into tri-methyl status in *hDAB2IP* gene promoter region.

On the other hand, for the methylation status of H3-K9, we failed to detect any good correlation among three cell lines tested. For example, tri-methyl H3-K9 was highly detectable in these cell lines and di-methyl K9 status was not consistent with hDAB2IP levels among these three cell lines (Figure 4D). Moreover, increased *Ezh2* expression in PZ-HPV-7 cells was accompanied with dramatic elevation of di-methyl but with not tri-methyl H3-K9 elevated dramatically (Figure 5D), suggesting that the methylation status of H3-K9 is not consistent with *hDAB2IP* gene promoter activity modulated by Ezh2. Together, we believe that *hDAB2IP* gene silencing in PCa can be mediated by the elevated levels of Ezh2 with hypermethylation of H3-K27 and the recruitment of HDAC, which provides an underlying mechanism of the functional role of Ezh2 in metastatic PCa.

DISCUSSION

PCa is a leading cause of cancer-related death in males and is second only to lung cancer. Although effective surgical and radiation treatments are available for clinically localized PCa, metastatic PCa remains essentially incurable. To understand the biology of metastatic PCa, data from a cDNA microarray analysis indicate that elevated Ezh2 possessing HMKTase activity is often associated with metastatic PCa but not with benign tissue and organ-confined tumor (23). Noticeably, clinically localized prostate cancers expressing higher levels of Ezh2 often have a poorer prognosis (24), suggesting that Ezh2 is not only a potential biomarker for predicting the relative risk of PCa progression but also a key contributor for the disease progression. Here we have demonstrated that *Ezh2* transcript and protein were consistently elevated in metastatic PCa cells than in normal cells. The pattern of *hDAB2IP* gene expression exhibited an inverse correlation with that of Ezh2, which prompted us to study the role of Ezh2 in regulating *hDAB2IP* gene expression since silencing *hDAB2IP* gene expression is mainly due to epigenetic regulation (2, 6, 7). Increased hDAB2IP levels were detected in PCa cells transfected with Ezh2 siRNAs (Figure 2). In contrast, the reduced *hDAB2IP* gene expression was observed in normal prostatic epithelial cells transfected with Ezh2 expression vector (Figure 3). These results provide an important mechanism of elevated Ezh2 during PCa progression.

In Drosophila, ESC-E(Z) proteins recognize and exert their activity through specific DNA sequences known as Polycomb Response Elements (38-40). In mammals, such elements have not been identified yet. It remains unclear how mammalian PcG complexes are recruited to chromatin to regulate expression of specific target gene. Because none of the HKMT-containing PcG complexes contain a site-specific DNA-binding protein, target genes cannot be identified by searching the consensus-binding site in the genome

(41). Thus, the ChIP assay can provide direct evidence for the possible interaction between Ezh2 complex and promoter region of any known gene. (42). In this study, our data indicate that the levels of Ezh2 associated with *hDAB2IP* promoter region are consistent with the steady-state levels of *hDAB2IP* mRNA and protein in all three cell lines tested. Most importantly, Ezh2 plays an active role in recruiting other PcG components such as Eed and Suz12 to form a functional complex along with HDAC1 to the promoter region of *hDAB2IP* gene. Based on these results, one could predict that the presence of elevated Ezh2 levels in PCa signifies the down regulation of tumor suppressor genes via the cooperative effect of histone methylation and deacetylation. This study provides further evidence to support the critical role of epigenetic gene regulation in PCa progression.

The term "epigenetic" refers to the heritable changes in gene expression that are caused by mechanisms other than the alteration in the nucleotide sequence. This concept generates tremendous new knowledge in understanding gene expression in mammalian cells. In addition to DNA methylation, increasing studies have focused on the impact of covalent modifications of the histone core in the nucleosome structure on gene regulation. One of the covalent modifications, lysine methylation, has emerged as an important player in regulating gene expression and chromatin function. Lysine methylation usually occurs on K4, 9, 27, 36 and 79 of H3, K20 of H4 and K26 of H1 (35, 36, 43) Biochemical and genetic studies indicate that methylation of different lysine residues, with the exception of H3-K79, is catalyzed by different proteins containing SET domain (44). Although Ezh2 protein contains a SET domain and several groups (10,11, 21) have shown Ezh2 with HMTase activity, its substrate specificity is still controversial. In addition, methyl lysine in H3 can exist in mono-, di-, and tri-methyl status (35, 36) and the functional significance of these modifications is largely unknown. It appears that both di-, and tri-methyl H3-K27 are consistent with the presence of Ezh2 in the promoter region of *hDAB2IP* gene from both normal prostate epithelia and PCa cells. The presence of Ezh2 can dramatically increase the levels of di- and tri-methyl H3-K27 implying that hypermethylation of H3-K27 could be a key factor in silencing *hDAB2IP* gene expression. In summary, *hDAB2IP* gene silencing mediated by Ezh2 complex and HDAC provides a better understanding of the functional role of Ezh2 in metastatic PCa and perhaps in other cancer types such as breast cancer.

ACKNOWLEDGEMENTS

We thank Dr. Jenuwein for providing Ezh2 expression vector, Dr. Pienta for providing VCAP cell line and Jennifer Stanfield for editing this manuscript. This work was supported by grants from the Department of Defense (W81XWH-04-1-0222).

REFERECES

1. Chen, H., Pong, R. C., Wang, Z., Hsieh, J. T. (2002) *Genomics* **79,** 573-581
2. Chen, H., Toyooka, S., Gazdar, A. F., Hsieh, J. T. (2003) *J. Biol. Chem.* **278,** 3121-3130
3. Wang, Z., Tseng, C. P., Pong, R. C., Chen, H., McConnell, J. D., Navone, N., Hsieh, J. T. (2002) *J. Biol Chem.* **277,** 12622-12631

4. Zhang, H., Zhang, R., Luo, Y., D'Alessio, A., Pober, J. S., Min, W. (2004) *J. Biol Chem.* **279,** 44955-44965
5. Zhang, R., He, X., Liu, W., Lu, M., Hsieh, J.T., Min, W. (2003) *J. Clin. Invest.* **111**, 1933-1943
6. Dote, H., Toyooka, S., Tsukuda, K., Yano, M., Ouchida, M., Doihara, H., Suzuki, M., Chen, H., Hsieh, J. T., Gazdar, A. F., Shimizu, N. (2004) *Clin. Cancer. Res.* **10,** 2082-2089
7. Yano, M., Toyooka, S., Tsukuda, K., Dote, H., Ouchida, M., Hanabata, T., Aoe, M., Date, H., Gazdar, A. F., Shimizu, N. (2005) *Int. J. Cancer.* **113,** 59-66
8. Kuzmichev, A., Jenuwein, T., Tempst, P., Reinberg, D. (2004) *Mol. Cell.* **14,** 183-193.
9. Cao, R., Zhang, Y. (2004) *Curr. Opin. Genet. Dev.* **14,** 155-164
10. Kuzmichev, A., Nishioka, K., Erdjument-Bromage, H., Tempst, P., Reinberg, D. (2002) *Genes. Dev.* **16,** 2893-2905
11. Cao, R., Wang, L., Wang, H., Xia, L., Erdjument-Bromage, H., Tempst, P., Jones, R. S., Zhang, Y. (2002) *Science* **298,** 1039-1043
12. Pasini, D., Bracken, A. P., Jensen, M. R., Denchi, E. L., Helin, K. (2004) *EMBO. J.* **23,** 4061-4071
13. Cao, R., Zhang, Y. (2004) *Mol. Cell.* **15,** 57-67
14. Jacobs, J.J., van Lohuizen, M. (2002) *Biochim. Biophys. Acta.* **1602,** 151-161
15. O'Carroll, D., Erhardt, S., Pagani, M., Barton, S. C., Surani, M. A., Jenuwein, T. (2001) *Mol. Cell. Biol.* **21,** 4330-4336
16. Plath, K., Fang, J., Mlynarczyk-Evans, S. K., Cao, R., Worringer, K. A., Wang, H., de la Cruz, C. C., Otte, A. P., Panning, B., Zhang, Y. (2003) *Science* **300,** 131-135
17. Silva, J., Mak, W., Zvetkova, I., Appanah, R., Nesterova, T. B., Webster, Z., Peters, A. H., Jenuwein, T., Otte, A. P., Brockdorff, N. (2003) *Dev. Cell.* **4,** 481-495
18. Kelly, W. G., Schaner, C. E., Dernburg, A. F., Lee, M. H., Kim, S. K., Villeneuve, A. M., Reinke, V. (2002) *Development* **129,** 479-492
19. Fong, Y., Bender, L., Wang, W., Strome, S: (2002) *Science* **296,** 2235-2238
20. Rossant, J. (2001) *Harvey Lect.* **97,** 17-40
21. Czermin, B., Melfi, R., McCabe, D., Seitz, V., Imhof, A., Pirrotta, V. (2002) *Cell* **111,** 185-196.
22. Muller, J., Hart, C. M., Francis, N. J., Vargas, M. L., Sengupta, A., Wild, B., Miller, E. L., O'Connor, M. B., Kingston, R. E., Simon, J. A. (2002) *Cell* **111,** 197-208
23. Varambally, S., Dhanasekaran, S. M., Zhou, M., Barrette, T. R., Kumar-Sinha, C., Sanda, M. G., Ghosh, D., Pienta, K. J., Sewalt, R. G., Otte, A. P., Rubin, M. A., Chinnaiyan, A. M. (2002) *Nature* **419,** 624-629
24. Rhodes, D.R., Sanda, M.G., Otte, A. P., Chinnaiyan, A. M., Rubin, M. A. (2003) *J. Natl. Cancer Inst.* **95**, 661-668
25. Kleer, C. G., Cao, Q., Varambally, S., Shen, R., Ota, I., Tomlins, S. A., Ghosh, D., Sewalt, R. G., Otte, A. P., Hayes,

D. F., Sabel, M. S., Livant, D., Weiss, S. J., Rubin, M. A., Chinnaiyan, A. M. (2003) *Proc. Natl. Acad. Sci. USA* **100,** 11606-11611

26. Raaphorst, F. M., Meijer, C. J., Fieret, E., Blokzijl, T., Mommers, E., Buerger, H., Packeisen, J., Sewalt, R. A., Otte, A. .P., van Diest, P. J. (2003) *Neoplasia.* **5,** 481-488
27. Tseng, C. P. Brent, D.E., Li, Y. M., Pong, R. C., and Hsieh, J. T. (1998) *Endocrinology* **139**, 3542-3553
28. Navone, N., Olive M., Ozen, M., Davis, R., Troncoso, P., Tu, S. M., Johnston, D., Pollack, A., Pathak, S., von Eschenbach, A.C., and Logothetis, C.J. (1997) *Clin. Cancer Res.* **3**, 2493-2500
29. Korenchuk, S., Lehr, J. E., Mclean, L., Lee, Y. G., Whitney, S., Vessella, R., Lin, D. L., Pienta, K. J. (2001) *In Vivo.* **15,** 163-168
30. Weijerman, P. C, Zhang, Y., Shen, J., Dubbink, H. J., Romijn, J.C., Peehl, D. M., Schroder, F. H. (1998).*Urology* **51,** 657-662
31. Weijerman, P. C., Konig, J. J., Wong, S. T., Niesters, H.G., Peehl, D. M. (1994) *Cancer Res.* **54,** 5579-5583
32. Sewalt R.G., Lachner, M., Vargas, M., Hamer, K.M., den Blaauwen, J.L., Hendrix, T., Melcher, M., Schweizer, D., Jennwen, T., Otte, A. P. (2002) *Mol. Cell. Biol.* **22,** 5539-5553
33. Bracken, A.P., Pasini, D., Capra, M., Prosperini, E., Colli, E., Helin, K. (2003) *EMBO. J.* **22,** 5323-5335
34. van der Vlag, J., Otte, A. P. (1999) *Nat. Genet.* **23,** 474-478
35. Peters, A. H., Kubicek, S., Mechtler, K., O'Sullivan, R. J., Derijck, A. A., Perez-Burgos, L., Kohlmaier, A., Opravil, S., Tachibana, M., Shinkai, Y., Martens, J. H., Jenuwein, T. (2003) *Mol. Cell.* **12,** 1577-1589
36. Paik, W. K., Kim, S. (1971) *Science* **174,** 114-119
37. Rice, J. C., Briggs, S. D., Ueberheide, B., Barber, C. M., Shabanowitz, J., Hunt, D. F., Shinkai, Y., Allis, C. D. (2004) *Mol. Cell.* **12,** 1591-1598
38. Sengupta, A. K., Kuhrs, A., Jürg Müller, J. (2004) *Development.* **131**, 1959-1965
39. Bloyer, S., Cavalli, G., Brock, H. W., Dura, J. M. (2003) *Dev. Biol.* **261,** 426-442
40. Pirrotta, V., Poux, S., Melfi, R., and Pilyugin, M. (2003) *Genetica* **117:** 191–197
41. Kirmizis, A., Bartley, S. M., Kuzmichev, A., Margueron, R., Reinberg, D., Green, R., Farnham, P. J. (2004) Genes Dev 18, 1592-1605.
42. Oberley, M.J., Tsao, J., Yau, P., and Farnham, P.J. (2004) *Methods Enzymol.* **376:** 316-335
43. Santos-Rosa, H., Schneider, R., Bannister, A.J., Sherriff, J., Bernstein, B.E., Emre, N.C., Schreiber, S.L., Mellor, J., Kouzarides, T. (2002). *Nature* **419,** 407–411
44. Sims, IIIrd. R.J., Nishioka, K., Reinberg, D. (2003) *Trends Genet.* **19**, 629–639

LEGENDS

Figure 1 Profiling *hDAB2IP* and *Ezh2* expression in prostatic epithelia. The steady-state levels of *hDAB2IP* (A) and *Ezh2* (B) mRNA from various prostatic epithelia cells were determined by qRT-PCR. After normalizing with 18S rRNA, the copy number of each sample (mean ± SD) performed in duplicates from two different experiments was calculated based on the standard curve. The steady-state levels of *hDAB2IP* and *Ezh2* protein (C) were determined by western blot analysis using the whole cell lysates extracted from four different prostatic epithelial cells.

Figure 2 The effect of Ezh2 on hDAB2IP protein expression in PCa cells.
The cell lysate from PC3 cells (A, B) or DU145 cells (C, D) was collected at the indicated time points after transfecting with either Ezh2 siRNA (A or C) or control Lamin A/C siRNA (B or D). The western blot analyses were performed using antibodies against Ezh2, hDAB2IP and LaminA/C. Actin was used as an internal loading control.

Figure 3 The effect of EZH2 on *hDAB2IP* promoter activity in normal prostatic epithelia. PZ-HPV-7 (A, B, D) and PrEC 1 (C, E) cells were transfected with both myc-tagged Ezh2 expression vector and pGL3-P2 reporter gene construct. In the time course experiment, Ezh2 expression vector (0.2 μg) and pGL3-P2 reporter gene construct (0.5μg) were used. A, an increased Ezh2 protein expression in PZ-HPV-7 cells. B and C,

the dose-dependent inhibition of *hDAB2IP* promoter activity by Ezh2. D and E, the time-dependent inhibition of *hDAB2IP* promoter activity by Ezh2.

Figure 4 The status of PcG complex, HDAC1, histone methylation and histone acetylation on the *hDAB2IP* promoter region in prostatic epithelia.

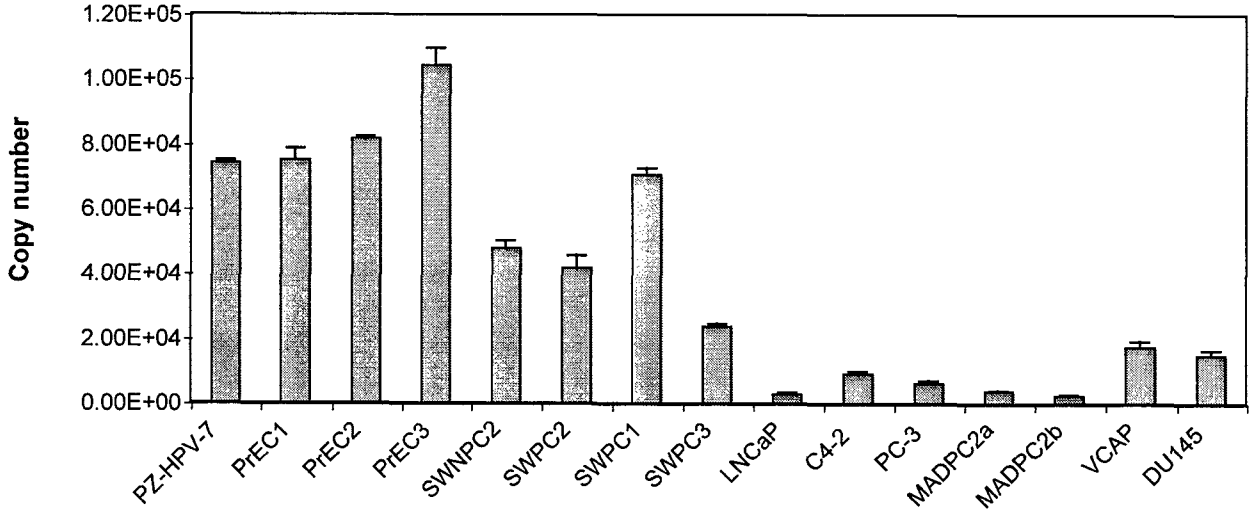
ChIP assay was performed from DNA-protein complex isolated from PZ-HPV-7, PC3 and DU145 cells and immunoprecipitated with various antibodies. The amount of *hDAB2IP* gene promoter was determined by qPCR using the specific primer set described previously (2) and visualized with gel electrophoresis. The number under each lane representing the fold of enrichment was calculated as $1/2^{\Delta Ct\,(sample)-\,\Delta Ct\,(PZ\text{-}HPV\text{-}7)}$.

Figure 5 The impact of Ezh2 on the status of H3 methylation and acetylation of *hDAB2IP* promoter region in PZ-HPV-7 cells after recruiting PcG complex proteins and HDAC1.

ChIP assay was performed from DNA-protein complex isolated from PZ-HPV-7 cells transfected with different amount of *Ezh2* expression vector and immunoprecipitated with various antibodies. The amount of *hDAB2IP* gene promoter was determined by qPCR using the specific primer set described previously (2) and visualized with gel electrophoresis. The number under each lane representing the fold of enrichment was calculated as $1/2^{\Delta Ct\,(Ezh2)-\,\Delta Ct\,(control)}$.

Fig.1

A


Copy number
1.20E+05
1.00E+05
8.00E+04
6.00E+04
4.00E+04
2.00E+04
0.00E+00
PZ-HPV-7
PrEC1
PrEC2
PrEC3
SWNPC2
SWPC2
SWPC1
SWPC3
LNCaP
C4-2
PC-3
MADPC2a
MADPC2b
VCAP
DU145

B

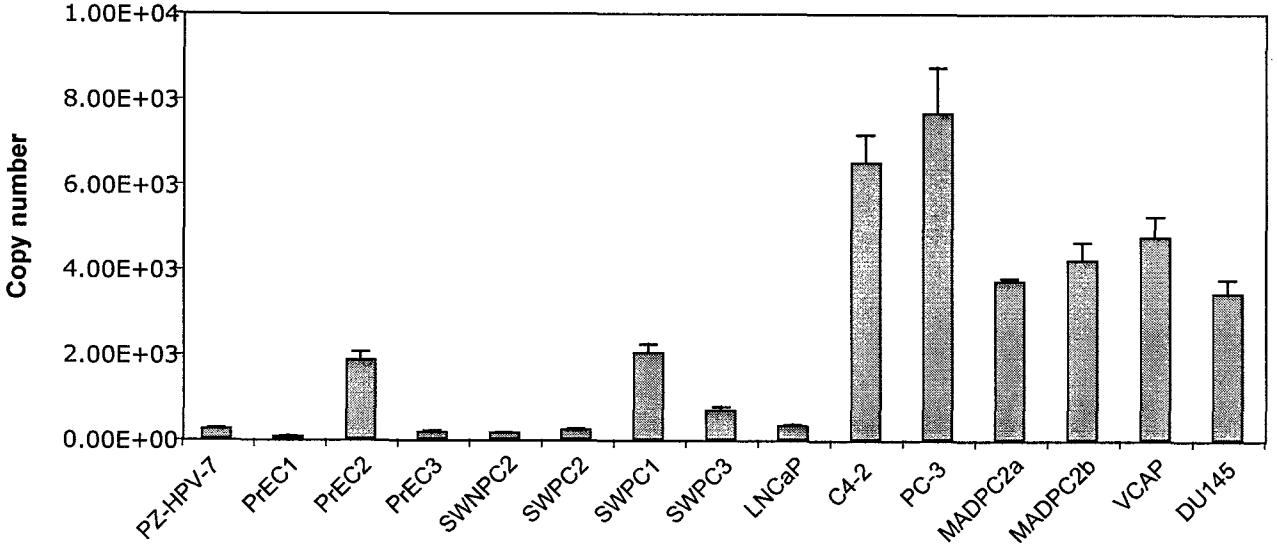

Copy number
1.00E+04
8.00E+03
6.00E+03
4.00E+03
2.00E+03
0.00E+00
PZ-HPV-7
PrEC1
PrEC2
PrEC3
SWNPC2
SWPC2
SWPC1
SWPC3
LNCaP
C4-2
PC-3
MADPC2a
MADPC2b
VCAP
DU145

Fig.1

C

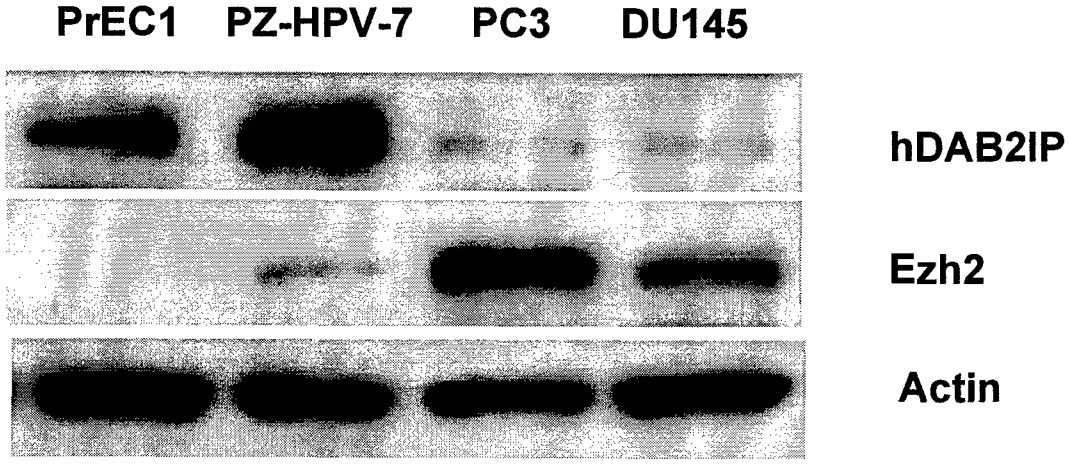


Fig.2

A

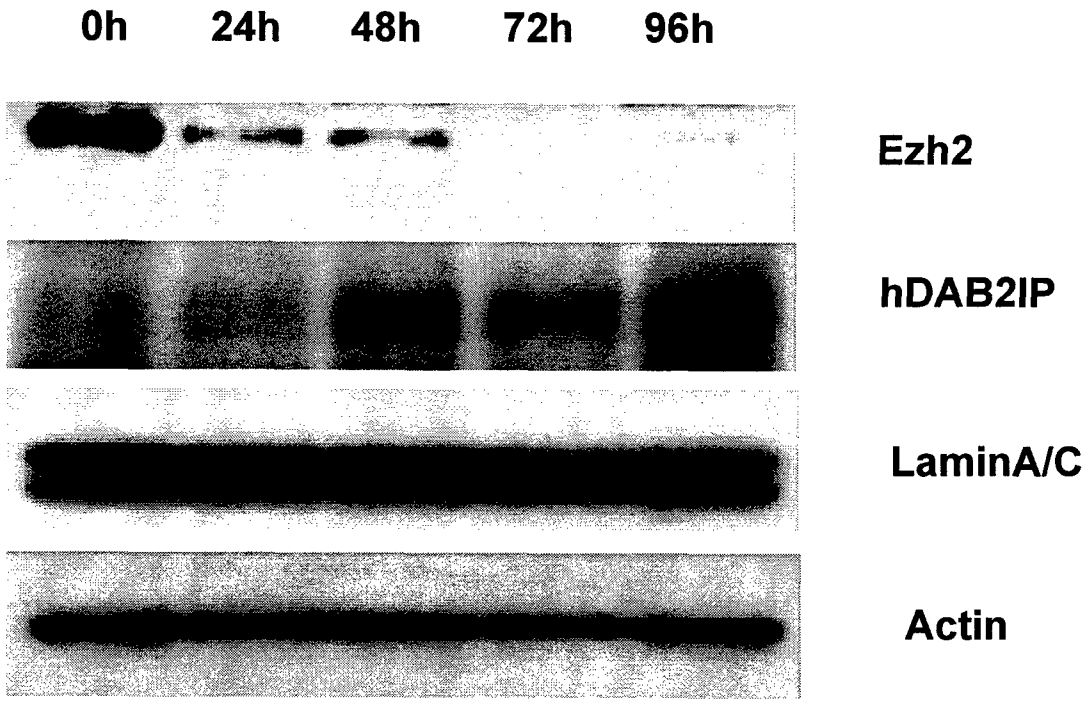


Fig.2

B

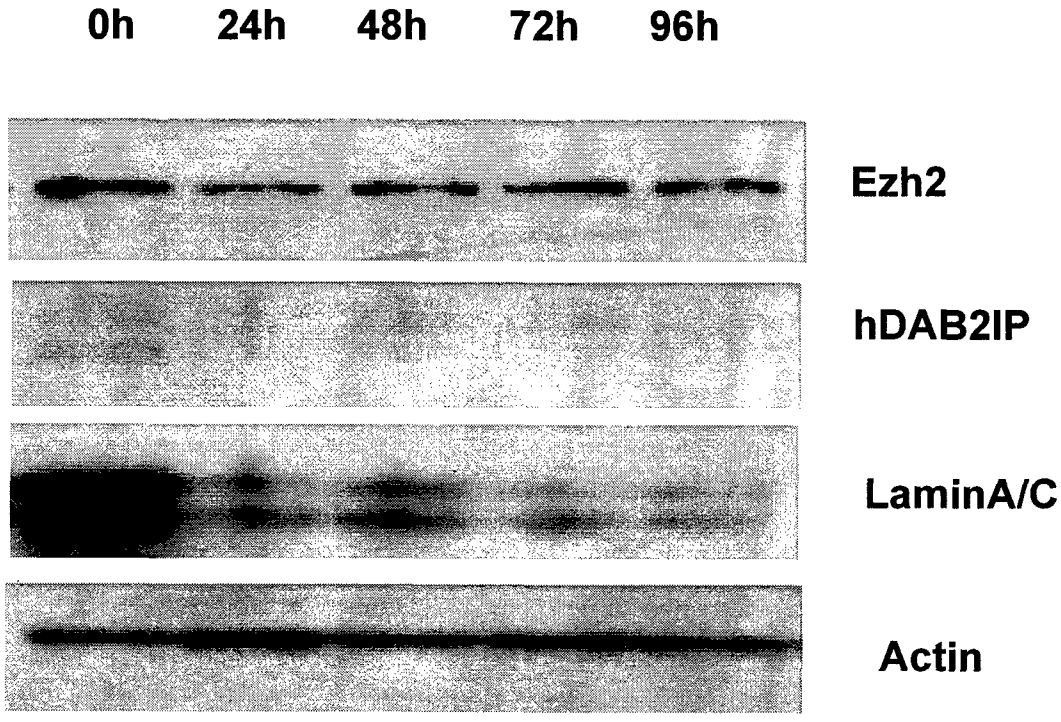


Fig.2

C

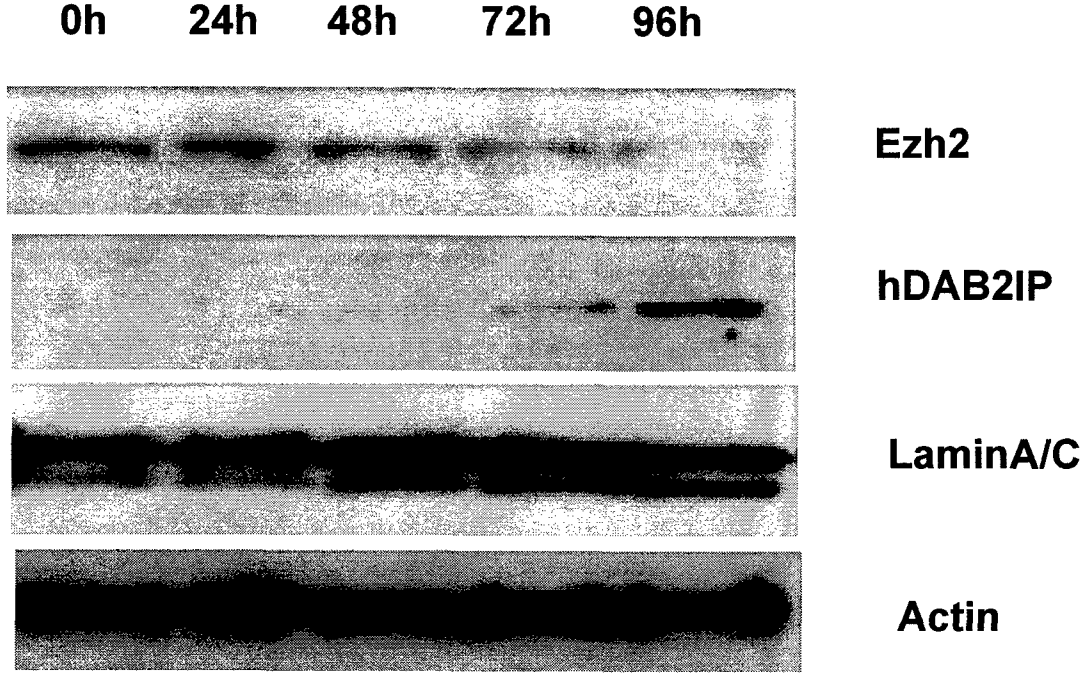


Fig.2

D

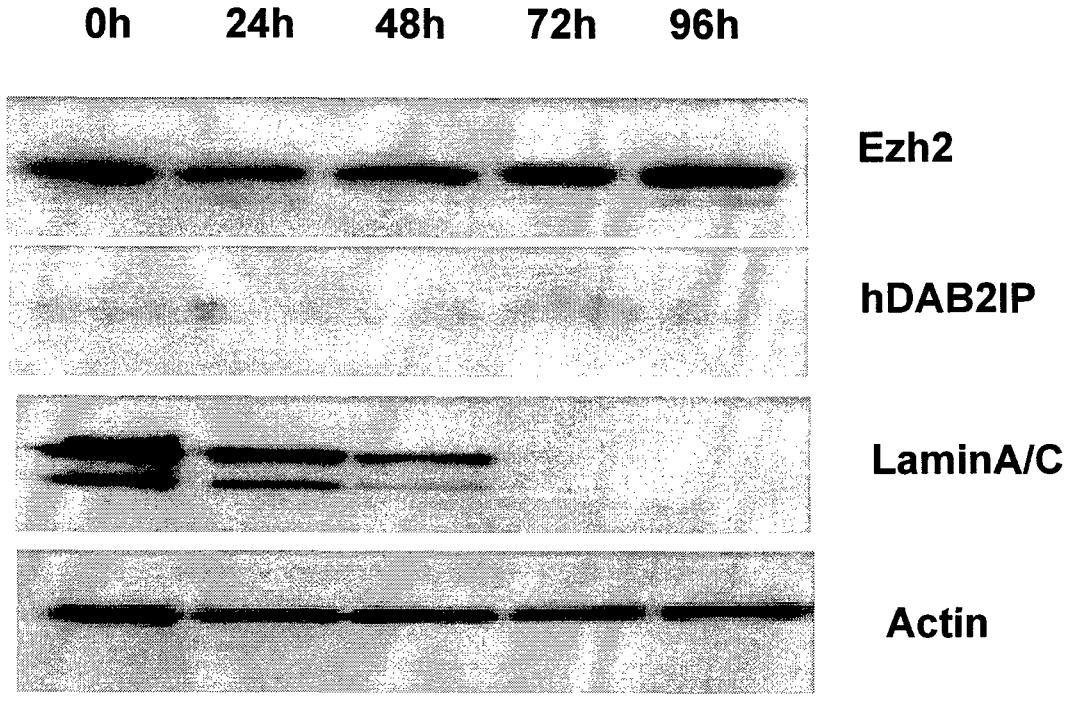


Fig.3

A

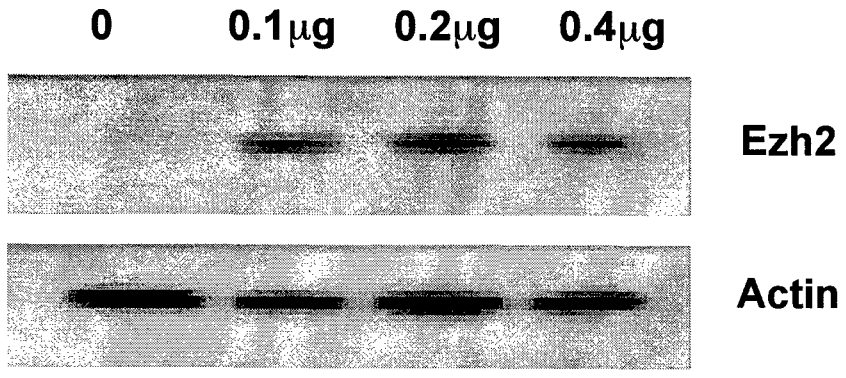
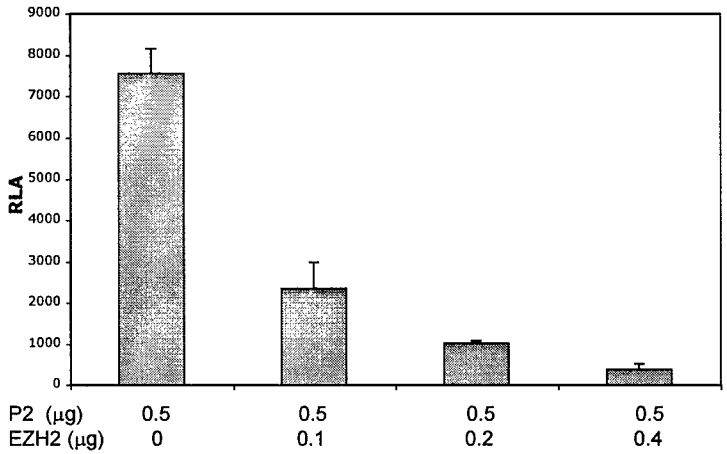
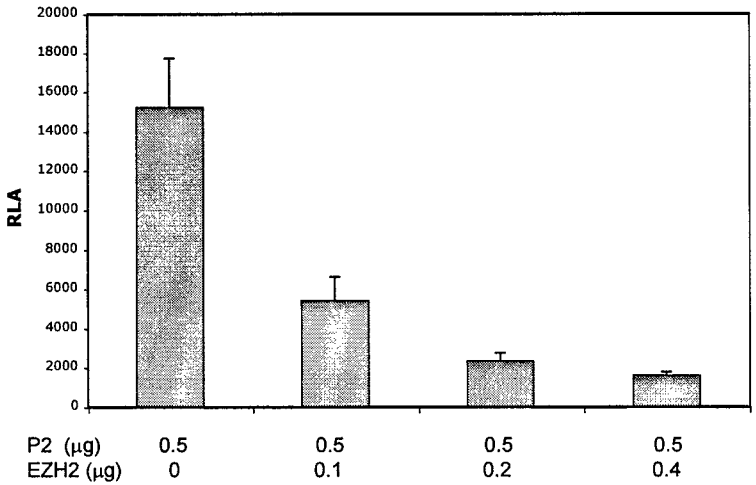


Fig.3

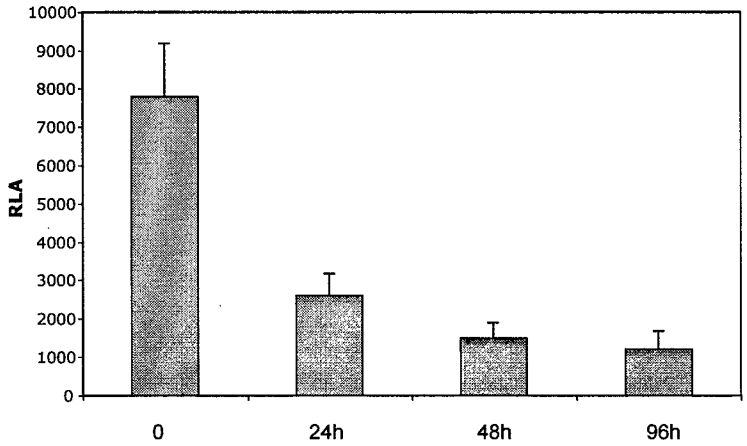
B


RLA
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
P2 (μg) 0.5 0.5 0.5 0.5
EZH2 (μg) 0 0.1 0.2 0.4

C


RLA
20000
18000
16000
14000
12000
10000
8000
6000
4000
2000
0
P2 (μg) 0.5 0.5 0.5 0.5
EZH2 (μg) 0 0.1 0.2 0.4

D


RLA
10000
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
0
24h
48h
96h

E

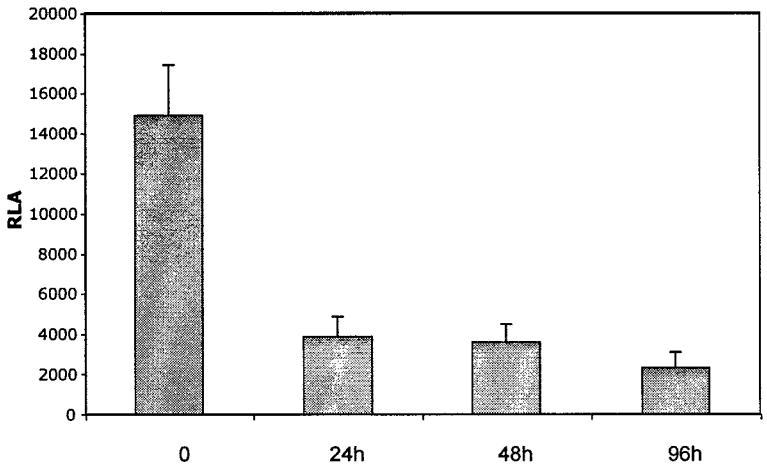

RLA
20000
18000
16000
14000
12000
10000
8000
6000
4000
2000
0
0
24h
48h
96h

Fig.4

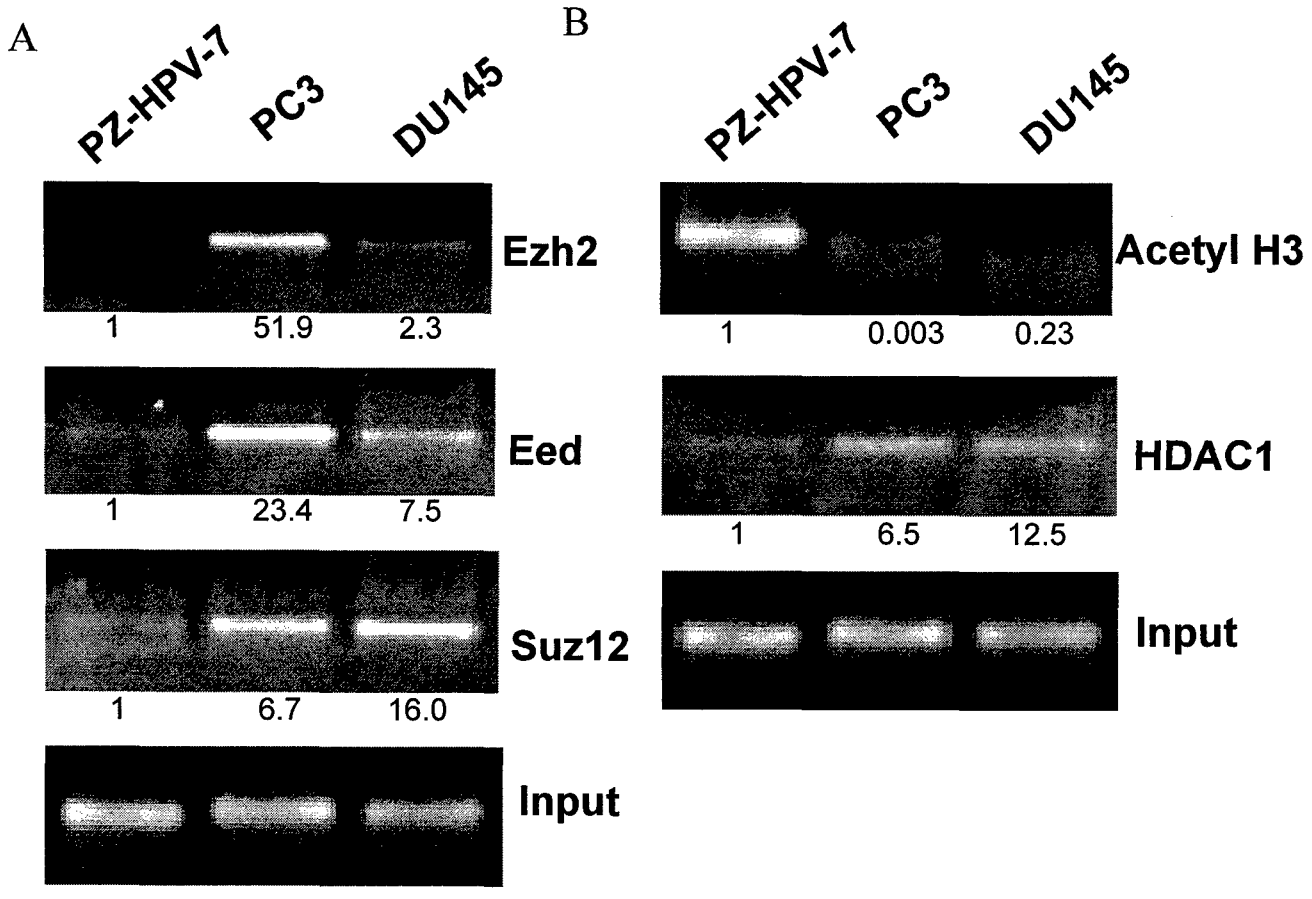

A
PZ-HPV-7
PC3
DU145
Ezh2
1
51.9
2.3
Eed
1
23.4
7.5
Suz12
1
6.7
16.0
Input
B
PZ-HPV-7
PC3
DU145
Acetyl H3
1
0.003
0.23
HDAC1
1
6.5
12.5
Input

Fig.4

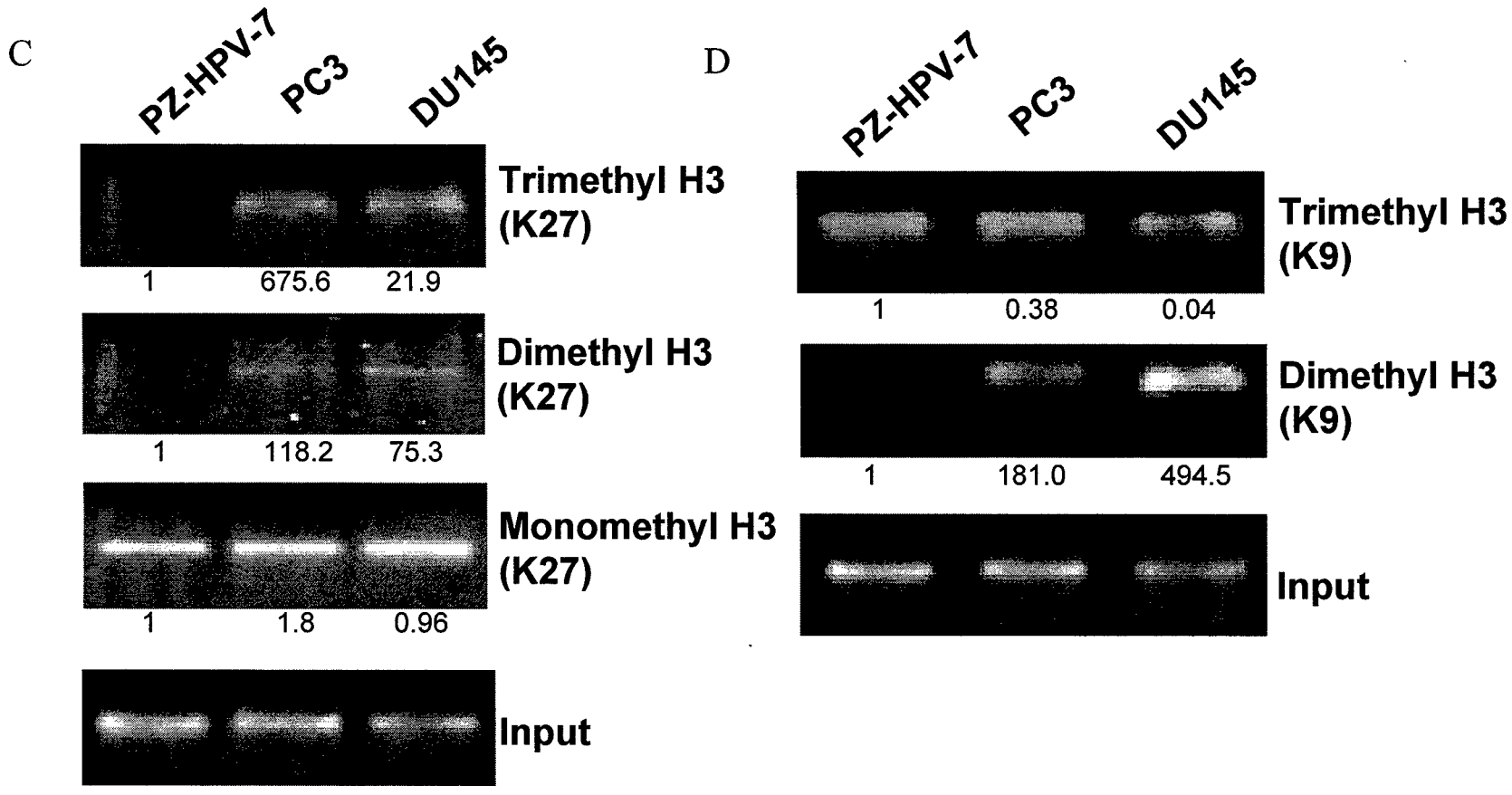

C
PZ-HPV-7
PC3
DU145
Trimethyl H3 (K27)
1
675.6
21.9
Dimethyl H3 (K27)
1
118.2
75.3
Monomethyl H3 (K27)
1
1.8
0.96
Input
D
PZ-HPV-7
PC3
DU145
Trimethyl H3 (K9)
1
0.38
0.04
Dimethyl H3 (K9)
1
181.0
494.5
Input

Fig.5

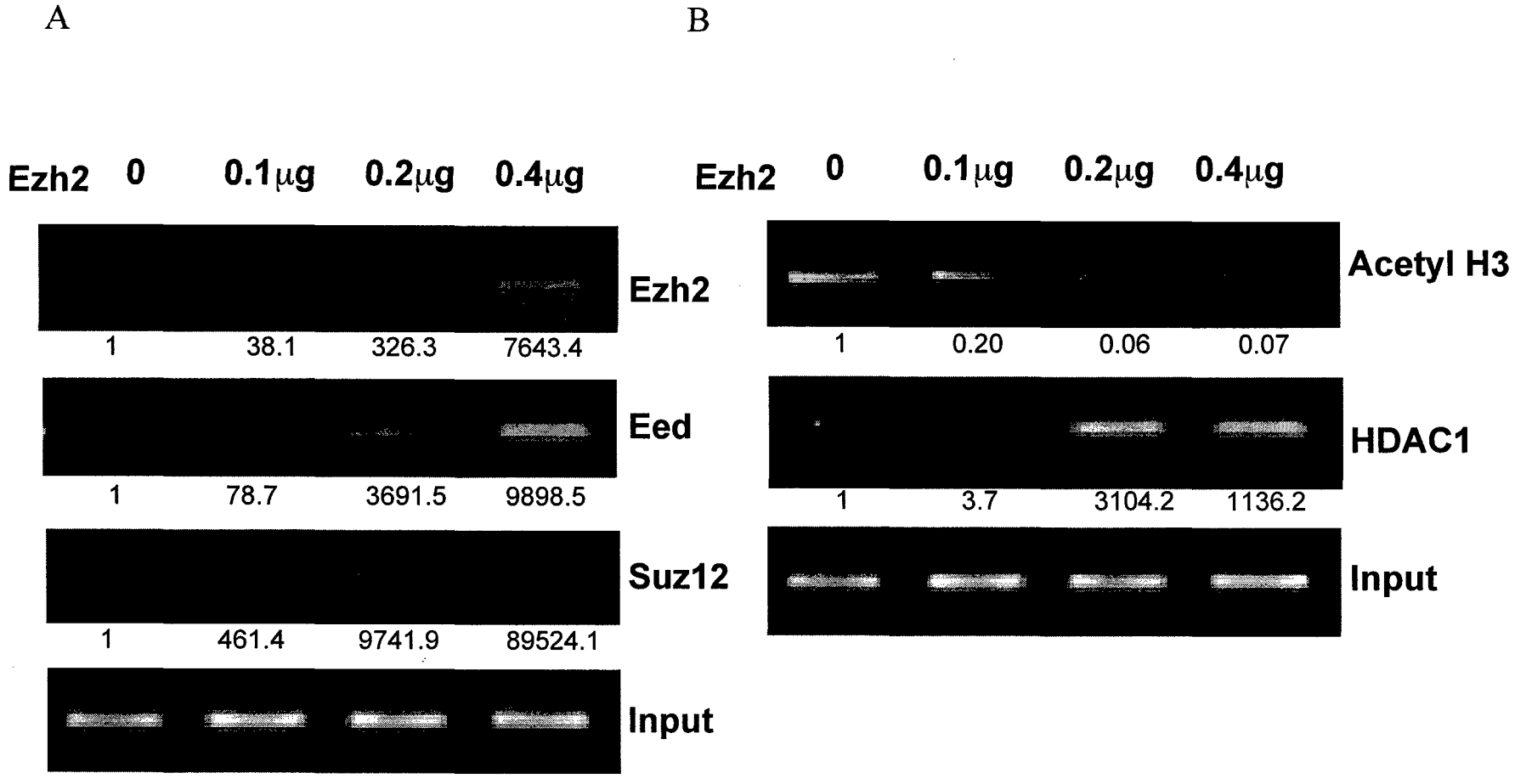
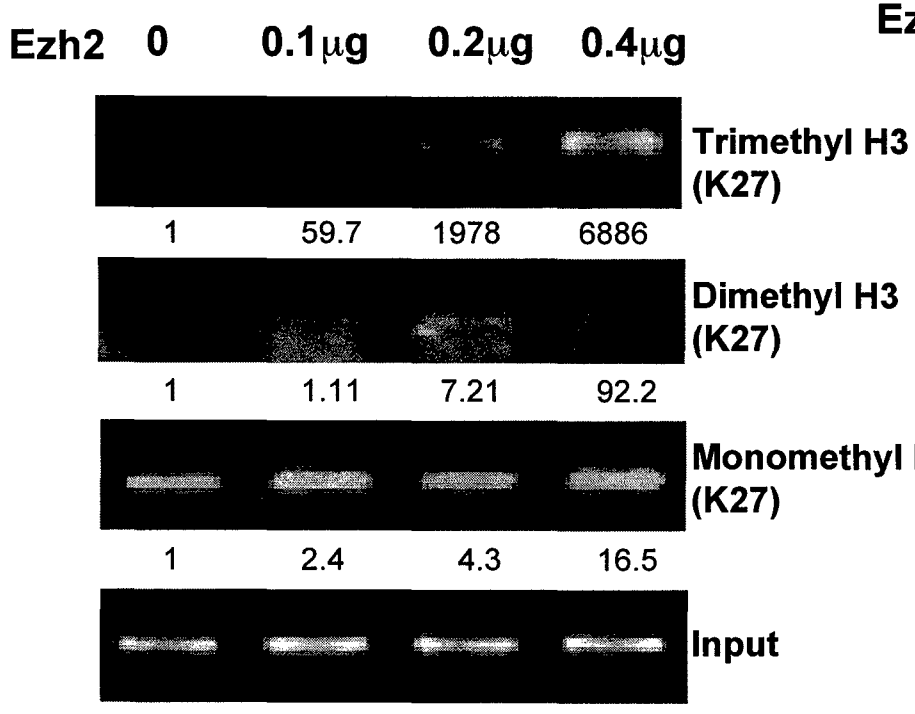
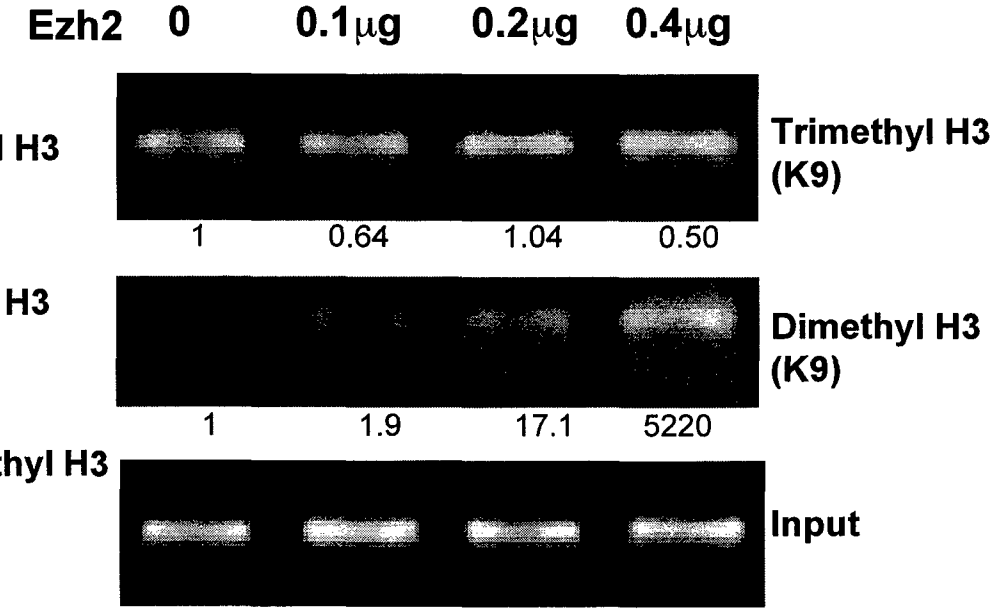

A
Ezh2 0 0.1μg 0.2μg 0.4μg
Ezh2
1 38.1 326.3 7643.4
Eed
1 78.7 3691.5 9898.5
Suz12
1 461.4 9741.9 89524.1
Input
B
Ezh2 0 0.1μg 0.2μg 0.4μg
Acetyl H3
1 0.20 0.06 0.07
HDAC1
1 3.7 3104.2 1136.2
Input

Fig.5

C


Ezh2 0 0.1μg 0.2μg 0.4μg
Trimethyl H3 (K27)
1 59.7 1978 6886
Dimethyl H3 (K27)
1 1.11 7.21 92.2
Monomethyl H3 (K27)
1 2.4 4.3 16.5
Input

D


Ezh2 0 0.1μg 0.2μg 0.4μg
Trimethyl H3 (K9)
1 0.64 1.04 0.50
Dimethyl H3 (K9)
1 1.9 17.1 5220
Input

Cloning of mouse Dab2ip gene, a novel member of the RasGTPase-activating protein family and characterization of its regulatory region

Hong Chen[1], Jose A Karam[1], Roger Schultz[2], Zhengwang Zhang[1], Christine Duncan[2] and Jer-Tsong Hsieh[1]

Department of Urology[1] and Pathology[2], University of Texas Southwestern Medical Center, Dallas, TX 75390-9110 (USA)

* Address all correspondence to:
J.T. Hsieh, Ph.D.
University of Texas Southwestern Medical Center
Department of Urology
5323 Harry Hines Blvd.
Dallas, TX 75390-9110
(Phone) 214-648-3988
(Fax) 214-648-8786
(E-mail) JT.Hsieh@UTSouthwestern.edu

Sequence data from this article have been deposited into the EMBL/GenBank Data Libraries under accession number AY305656, AY305657 andAY305658

Key words: **Dab2ip gene, prostate cancer, gene promoter**

Abstract

Disabled homolog 2 (Drosophila) interacting protein (DAB2IP) is a novel member of GTPase-activating protein for down-regulating the Ras-mediated signal pathway. The down regulation of hDAB2IP mRNA levels were detected in prostate cancer cells due to the epigenetic regulation. Here, we isolated a mouse Dab2ip (mDab2ip) gene with a highly homologous sequence to that of the human and rat gene and mapped it at chromosome 2B. The mDab2ip gene contains 14 exons and 13 introns and spans approximately 65kb. Exon1 contains at least three variants, exon Ia; exon Ib and exon Ic. The deduced amino acid sequence of mDab2ip encompasses 1065 residues containing several unique protein interaction motif as well as a Ras-like GAP-related domain, which shares a high homology among human and rat. Data from real-time RT-PCR analysis revealed a diverse expression pattern of mDab2ip gene in various organs, implying that differential regulation of this gene from various tissues. We have mapped a 1.3kb segment containing 5'-upstream region from exon Ia as a promoter region (–147/+545) in prostatic epithelial cell lines (TRAMP-C); this region is highly GC-rich and mDab2ip appears to be a TATA-less promoter.

Introduction

Disabled homolog 2 (Drosophila) interacting protein (DAB2IP) is a novel member of the Ras GTPase-activity family protein (Chen et al., 2002; Wang et al., 2002) Our data indicated that DAB2IP interacted directly with DOC-2/DAB2 protein (Zhou and Hsieh, 2001; Fulop et al., 1998; Tseng et al., 1999; Zhou et al., 2003) and this protein complex modulated Ras-mediated signal pathway resulted in the growth inhibition in prostate cancer cells (Wang et al., 2002). Also, DAB2IP (also named AIP1: ASK interacting protein 1) was involved in TNF-α-mediated cell apoptosis by facilitating dissociation of ASK1 from its inhibitor 14-3-3 (Zhang et al., 2003)

The higher hDAB2IP mRNA levels were detected in normal human prostatic epithelium than in prostate cancer cells, which was due to the epigenetic regulation (Jones et al., 2001; Wolffe and Matzke 1999) such as DNA methylation and histone acetylation) of human DAB2IP (hDAB2IP) gene promoter (Chen et al., 2002; Chen et al., 2003). In breast cancer, DNA hypermethylation of hDAB2IP was also found in both breast cancer cell lines and specimens with lymph node metastasis, and *hDAB2IP* gene expression could be restored in methylated cell lines treated with 5-aza-2′-deoxycytidine (Dote et al., 2004). Also, hDAB2IP (alias for AFQ34) was identified as a novel MLL fusion partner from an acute myeloid leukemia patient with a t(9;11)(q34;q23). In this case, the intron 9 of MLL gene was translocated into the exon 2 of DAB2IP resulted in the disruption of pleckstrin homology (PH) domain in DAB2IP protein, implying that this fusion protein may disable the regulation of RAS activity as a part of leukemic

transformation process (von Bergh et al., 2004). Thus, DAB2IP should be involved in carcinogenesis of various tissues.

To further unveil the physiological functions of mouse Dab2ip (mDab2ip), we decided to clone and map its chromosomal location. With analyzing the structure of mDab2ip gene, we were able to constitute its full-length mRNA and open reading frame. We also profiled the mDab2ip expression pattern from a variety of organs. By determining the promoter sequence from the 5'- flanking region of the mDab2ip gene in mouse prostatic epithelial cell lines provided clues for the transcriptional regulation of mDab2ip gene.

Materials and Methods

Tissue Culture and RNA isolation

Three mouse transgenic prostate adenocarcinoma cell lines (TRAMP-C1, TRAMP-C2 and TRAMP-C3) (Foster et al., 1997; Greenberg et al., 1995; Gingrich et al., 1997) were maintained in DMEM supplemented with 5% FBS (HyClone, Logan, UT) plus 5% Nu-serum^{TM} IV (BD Bioscience, Bedford, MA) and 50ng/ml insulin (Sigma). Total RNA from organs and from three TRAMP-C lines were isolated using the RNAzol B (Tel-Test Inc., Friendswood, TX) according to the manufacturer's instructions.

For tissue RNA isolation, various organs (approximate 50 mg) were harvested from nude mice after euthanasia and were snap-frozen in liquid nitrogen until analysis was performed. Tissues were submerged with RNAzol B and quickly homogenized then subjected to the same isolation procedure.

Cloning mDab2ip gene and sequence Analysis

To obtain the entire coding region of mDab2ip cDNA, we performed RT-PCR from total cellular RNA from mouse brain. Based on the high homology sequences between human and rat *DAB2IP* cDNA [1, 2], two sets of primer were synthesized (Table 1). PCR products were cloned into pCR 2.1-TOPO vector (Invitrogen) and sequenced then used as probes for screening mDab2ip gene.

A mouse bacterial artificial chromosomal (BAC, RPCI.22) library (ResGen Invitrogen Corp. Huntsville, AL) was screened. The positive clones were subjected to Southern blot analysis and DNA sequencing for confirmation.

Identification of transcriptional starting site (TSS) by 5' RACE

To determine the transcriptional starting site of mDab2ip gene, The total cellular RNA (10 μg) from two mouse organs (brain and kidney) and two TRAMP-C lines (TRAMP-C1 and TRAMP-C2) was subjected to 5' RACE using the FirstChoice™ RLM-RACE Kit (Ambion Inc, Austin, TX) according to the manufacturer's manual. A random-primed reverse transcription reaction and nested PCR (Fig 2B and Table1) were performed to amplify the 5'end of the mDab2ip mRNA transcript. We analyzed the PCR product in a 2% NuSieve® 3:1 agarose gel (Cambrex BioScience, Rockland, MA) and then cloned it into pCR2.1-TOPO for sequence identification.

Determination of mDab2ip mRNA levels by Real-time RT-PCR assay

Two micrograms of total cellular RNA were reversely transcribed into cDNA and amplified using either mDab2ip primer set (2 ng/μl) or *Actin* primer set (6 ng/μl) (Table 1) in a 40-μl reaction mixture containing 20-μl IQ™ SYBR® Green Supermix (Bio-Rad Laboratories, Hercles). The PCR was performed using iCycler machine (Bio-Rad) and the reaction condition was as follow: 95°C (3 min) and 40 cycles amplification cycle (95°C [30 sec], 55°C [30 sec], and 72°C [1 min]). To assure the quality of each reaction, melting curves analysis was performed using 95°C (1 min), 55°C (1 min) and 80 cycles of 0.5°C increment beginning at 55°C. Each sample was performed in duplicates. The level of mDab2ip mRNA from each organ was calculated as follows: ΔC_t (threshold cycle) of each sample = mean of $C_{t(\text{Dab2ip})}$ - mean of $C_{t(Actin)}$. The relative expression of mDab2ip in each organ was calculated as $1/2^{\Delta C_{t(\text{tissue})} - \Delta C_{t(\text{spleen})}}$ since spleen had the lowest Dab2ip mRNA level. Meanwhile, The levels of mDab2ip mRNA from each TRAMP-C

were calculated as $1/2^{\Delta C_{t(\text{TRAMP-C})} - \Delta C_{t(\text{TRAMP-C3})}}$ since TRAMP-C3 had the lowest expression of mDab2ip.

Fluorescence in Situ Hybridization (FISH) analysis

To determine the chromosomal localization of the mDab2ip gene, cells were sterilely isolated from the spleens of 6-week old mice and set up in RPMI 1640 with glutamine, 20% FBS, and 50μg lipopolysaccharide at 37°C for 42 hours. At 42 hours 0.75μl of 10μg/ml colcemid was added and incubated for 10 minutes. Cells were pelletted, resuspended in 1 ml of hytotonic KCl (prewarmed to 37°C) and incubated at room temperature for 15 minutes. The cells were then fixed in methanol:acetic acid (3:1) and spread on slides using heat treatment. DNA probes from BAC clones 22N15 and 301E21 were fluorescently labeled by nick translation using standard conditions. Probe was hybridized to mouse metaphase slides overnight on a HYBrite (Vysis, Inc., Downers Grove, IL) hybridization chamber and washed. Hybrdization signal was viewed and analyzed on an Olympus AX70 fluorescence microscope and images captured using MacProbe software (version 4.4, Applied Imaging).

Construction of luciferase reporter plasmid containing the 5'-upstream regulatory sequence of mDab2ip gene

To analyze the 5'-upstream regulatory sequence of the mDab2ip gene, a 1.3kb fragment from –730 to +545 (transcription initial site as +1 predicted by 5'RACE data) containing upstream region, exon Ia and partial intron 1a was amplified by PCR from clone 22N15. To further define the promoter region in mDab2ip gene, a series of deletion

mutants were generated by PCR (Table 1). The PCR products were subcloned into pCR®-Blunt II TOPO vector (Invitrogen). After sequencing confirmation, they were further cloned into pGL3 basic vector (Promega) using KpnI/XhoI sites to generate pGL3-F1/R2 (from –730 to +545), pGL3-F6/R2 (form-421 to +545), pGL3-F8/R2 (from +6 to +545), pGL3-F10/R2 (from +249 to +545), pGL3-F12/R2 (from +445 to +545), The pGL3-F7/R2 contains a NcoI fragment (from –147 to +545) of mDab2ip. The pGL3-F6/Nco I contains a 0.3 kb insert from –421 to -157 and pGL3-F6/SanD I contains a 0.4 kb insert from-421 to -97.

Measurement of mDab2ip putative promoter activity using reporter gene assay

Both TRAMP-C1 and TRAMP-C3 were plated at a density of 0.6×10^5 cells per well in a 6-well plate. After 16 hrs, cells were transfected with both 0.8 μg of reporter vectors and 0.2 μg β-galactosidase vector (pCH110) using Lipofectamine Plus transfection reagent (Invitrogen). Forty-eight hours after transfection, cells were washed twice with cold phosphate-buffered saline (PBS) and harvested them in Lysis Buffer (Promega) then cell lysate was subjected to luciferase and β-galactosidase (β-gal) assays as described previously (Chen et al., 2003). The relative luciferase activity (RLA) from each sample was determined by normalizing the luciferase activity with its β-gal activity. All experiments were repeated at least three times in triplicate.

Result

Characterization of mDab2ip gene

A mouse bacterial artificial chromosomal (BAC) library was screened with two partial mDab2ip cDNA probes. Three positive clones were identified (22N15, 301E21, and 538A16). Two of them (22N15 and 301E21) were chosen for further study because both contained mDab2ip gene confirmed by PCR and Southern blot analysis using a 800-bp partial mDab2ip cDNA probe (data not shown). We performed sequencing analysis using Sp6 and T7 primers to analyze both clones. With BLAST program (NCBI, http://www.ncbi.nlm.nih.gov), we matched these two clones with the Mus musculus chromosome 2 genomic contig sequence (accession no. NT_039206). The sequence data showed the 3'end sequencing (T7 primer) of clone 22N15 aligned with the middle portion of the mDab2ip gene and the 5' end sequence (Sp6 primer) aligned with 5' upstream of NT_039206. The sequence of clone 301E 21 spans the entire mDab2ip gene except 5' upstream regulation region (Fig. 1A). Furthermore, we performed FISH analysis using 22N15 and 301E21 as probes, we were able to locate mDab2ip gene at chromosome 2B (Fig. 2A and B).

We deduced the exon-intron junction of mDab2ip by aligning its cDNA sequence with NT_039206. Furthermore, we confirmed all predicted exon–intron boundaries by PCR and DNA sequencing, which junction coincides with the GT...AG rule. It appears that mDab2ip gene contains 14 exons and 13 introns (Fig. 1A and Tables 2 and 3). Noticeably, exon 1 is a non-coding exon that was separated from exon 2 by a large intron (>10 kb). The translation initiation site (ATG) is mapped at the 11-bp downstream from

the 5'-end of exon 2 and the protein termination site (TAA) is located at exon 14 followed with a large untranslated sequence.

To further determine the transcription starting site(s) of mDab2ip gene, we designed two mDab2ip specific primers (Sp1, Sp2) to combine with universal outer and inner adapter primers for nested PCR (Fig. 1B). As show in Fig. 1C, two PCR transcripts (300 bp and 600 bp) were detected from RNA isolated from mouse brain and three mouse prostatic epithelial cell lines (TRAMP-C1, -C2 and –C3). In contrast, only one single transcript (300 bp) was detected from kidney RNA. DNA sequencing data revealed at least three variants form the exon 1. The mDab2ip mRNA from mouse brain and TRAMP-C contains both exon Ia and exon Ib; the mDab2ip mRNA from mouse kidney contains exon Ic.

A high homology of deduced protein sequence among mouse Dab2ip, human and rat DAB2IP

In our recent study (Chen et al., 2002), we show that the deduced DAB2IP protein sequence is remarkably similar between rat and human (94% homology). To obtain the entire open reading frame of mDab2ip, we performed RT-PCR from RNA isolated from mouse brain using primer sets based on rat *DAB2IP* cDNA sequence (Chen et al., 2002; Wang et al., 2002). The deduced 1065 amino acids of mDab2ip revealed a high homology sequence with human and rat counterpart (Fig. 3A). Using the program "NCBI conserved domain database " (http://www.ncbi.nih.gov/structure/cdd/wrpsb.cgi), ScanProsite program (http://au.expasy.org/cgi-bin/scanprosite) and Motif Scan Graphic program (http://scansite.mit.edu), there are five conserved protein domain predicted: PH (pleckstrin homology domain 30-79), C2 (protein kinase C conserved region2 [CalB,

amino acid 90-189), RasGAP (GTPase-activating protein, amino acid 212-539), proline-rich domain (amino acid 796-805), and a leucine zipper domain (amino acid 911-932) (Fig. 3B). These data indicate that mDab2ip appear to be a new member of RasGAP family protein.

Expression profile of mDab2ip

To determine the tissue distribution of mDab2ip mRNA, we performed a real-time RT-PCR analyses (Fig. 4A). Results indicated a unique expression pattern in certain organs. For example, mDab2ip was most abundant in brain (72.5-fold), salivary gland (38.7-fold) and testis (21.3 fold); moderate expression in kidney (15.0-fold) and heart (11.3-fold); low expression in lung (7.4-fold), seminal vesicle (7.1-fold), ventral prostate (6.5-fold), epididymis (6.1-fold), liver (5.9-fold) and bladder (5.6-fold); very lower expression in coagulation gland (3.6-fold) and skeleton muscles (2.0-fold) compared with spleen that has the lowest expression level (= 1.0) among all the organs tested. This pattern of expression is consistent with our previous report (Wang et al., 2002) and gene card expression pattern (http://bioinformatics.weizann.ac.il/cards-bin/arddisp?DAB2IP) except the expression level of Dab2ip detected from heart.

In established cell lines, we also observed differential expression level of mDab2ip transcript among three prostatic epithelial cells (TRAMP-C1, TRAMP-C2 and TRAMP-C3) using a real-time RT-PCR. In general, the highest level (2.5-fold) of mDab2ip transcript was detected in TRAMP-C1 line among three cell lines tested (Fig. 4B).

Analysis of the 5'-upstream sequence of the mDab2ip gene

To analyze the promoter region of the mDab2ip gene, a 1.3kb fragment from position –730 to +545 (transcription initial site as +1 predicted by 5'RACE) containing 5' upstream region of the exon Ia, exon Ia and partial intron1a region was amplified by PCR from clone 22N15 using primer set F1/R2 (Table 1). Sequencing analysis indicated that this region is very GC-rich. Using TFSEARCH program (http://www.cbrc.jp/research/TFSEARCH.html); Promoter Scan II program (http://bimas.dcrt.nih.gow/molbio/proscan) and MacVector 7.0 program, we identified several potential *trans*-factor binding sites (Fig. 5A) including Sp1, AP-1 AP-2, SREBP, and p300, GATA-1/2, PEA2, AML-1a and MalT_box. Neither TATA-box nor CAAT-box was identified. The similar *trans*-factor binding sites were also detected in *hDAB2IP* gene (Chen et al., 2002). It indicates that Dab2ip gene is a typical TATA-less promoter.

To define the potential promoter region in the mDab2ip gene, we examined the reporter gene activities of a series of deletion constructs (Fig. 5B) generated from the clone 22N15. Using both TRAMP-C1 and TRAMP-C3 cells (Fig. 5C and 5D), we observed both constructs (pGL3-F6/R2 [form -421 to +545] and pGL3-F7/R6 [from -147 to +545]) expressed higher levels of luciferase reporter gene than that of pGL3-F1/R2 (from –730 to +545), suggesting that the presence of a negative *cis*-element between -730 and –421. On the other hand, the reporter gene activity decreased significantly in the rest of deletion constructs (i.e., pGL3- F8/R2, pGL3- F10/R2, pGL3-F12/R2). Also, very little reporter gene activity was observed in cells transfected with either pGL3 F6/NcoI (from -421 to –157) or pGL3-F6/SanDI (from-421 to –97). We

therefore conclude that the basal promoter region of mDab2ip gene is between -147 and +545. Noticeably, a good correlation between the mDab2ip mRNA level and the reporter gene activity was observed in these TRAMP-C cells, indicating that this is a promoter operative in mouse prostatic epithelial cells.

Discussion

The mDab2ip gene spans approximately 65kb containing 14 exons and 13 introns with at least three variants: exon Ia, exon Ib and exon Ic found from different sources of RNA using 5'RACE assay. All three splicing sequences of mDab2ip cDNA have been submitted to the GenBank™ (AY305656 [mDab2ip a]; AY 305657 [mDab2ip b]; AY305658 [mDab2ip c]). Using FISH analysis, mDab2ip was localized at chromosome band 2B (Fig. 2), which is consistent with the LocusLink program analysis (http://www.ncbi.nih.gov/ LocusLink).

In this study, we performed a real-time RT-PCR to demonstrate the mDab2ip mRNA levels in different organs (Fig. 4A). Very abundant mDab2ip mRNA levels were found in brain, salivary gland and testis and the moderate levels were found in kidney and heart. In addition, organs such as lung, seminal vesicle, ventral prostate, epididymis, liver and bladder only express low levels of Dab2ip mRNA. Also, the lowest level of Dab2ip mRNA was detected in coagulation gland, skeletal muscles and spleen. Such diverse expression pattern of Dab2ip gene implies that mDab2ip may have a unique physiological function in specific organ. To gain the insight of the mechanisms of mDab2ip transcriptional regulation, we isolated ~1.3kb (Fig. 5A) fragment containing 5'-upstream region from exon Ia and we found very rich GC-rich sequences and no canonical TATA boxes in this region. Nevertheless, we have shown that the 5'-flanking region from positions –730/+545 could enhance the reporter gene activity and it contained the basal promoter (-147/+545) and a negative regulatory element (-730/-421) (Fig. 5). Furthermore, we also identified several putative *cis*-elements in this region that

could underlie the differential Dab2ip gene expression in various organs or cells. Further analyses are warranted.

In our previous publications (Chen et al., 2002; Wang et al., 2002), we found the transcription initiation site (ATG) of *DAB2IP* at 63-bp from the 5'-end of exon 3 and predicted a putative open reading frame encoding 967-amino acid for hDAB2IP or 996-amino acid for rat DAB2IP (rDAB2IP). However, the updated sequence data from NCBI (accession no. NP_619723) indicate that an additional ATG site is mapped at 11-bp from the 5'-end of exon 2 that was also detected in mDab2ip. Thus, predicted mDab2ip protein encodes 1065-amino acid containing an additional 69-amino acid with a PH domain. PH domain is a short motif that mediates membrane localization and is found in many proteins involved in signal transduction, including GAPs for Ras (Rebecchi et al., 1998; Shaw 1996). The predicted protein sequence alignment between mouse and human DAB2IP is remarkable conserved. Von Bergh (Von Bergh et al., 2004) reported that the *hDAB2IP* is the alias for *AF9Q34*gene (accession no. AY032952) as a novel fusion partner of MLL in AML patient with a t(9:11). The juxaposition of MLL intron 9 into exon 2 of AF9Q34 will result in the loss of the exon 2 splicing donor site. Consequently, the *hDAB2IP*/AF9Q34 exon 2 sequences will be spliced out and result in a MLL-exon 9/AF9Q34-exon 3 fusion product. In this case, the AF9Q34-MLL fusion protein does not contain PH domain, implying that the normal function of the AF9Q34 gene may be aborted due to the chromosomal translocation.

Sequence analysis of mDab2ip revealed the presence of a highly conserved GRD (GAP related domains), the catalytic unit to stimulate the GTPase activity of Ras proteins, in the N-terminus of mDab2ip. GRD is a characteristic domain in the all

RasGAPs such as human neurofiibromin (NF1), rat SynGAP, p120GAP and human nGAP (Glanzer et al., 2002; Li et al., 1996; Kim et al., 1998; Bernards et al., 1992; Davis et al., 1993; Noto et al., 1998). Homayouni *et al.* suggested that Dab2ip could function as a down stream effector in the Reelin-signaling pathway that influences Ras signaling during brain development (Homayouni et al., 2003). Thus, Dab2ip appears to be a critical RasGAP protein in various tissues.

Acknowledgments

We thank Dr. Norman Greenberg (Baylor College of Medicine) for providing TRAMP-C cell lines and Kenneth S. Koeneman (UT Southwestern Medical Center) for reading this manuscript. This work is supported in part by United States Army Grant DAMD17-03-2-0033 and PC030301.

References

Bernards A, Haase VH, Murthy AE, Menon A, Hannigan GE, Gusella JF: Complete human NF1 cDNA sequence: two alternatively spliced mRNAs and absence of expression in a meuroblastoma line. DNA Cell Biol 11: 727–734 (1992).

Chen H, Pong RC, Wang Z, Hsieh JT: Differential regulation of the human gene DAB2IP in normal and malignant prostatic epithelia: cloning and characterization. Genomics 79: 573-581 (2002).

Chen H, Toyooka S, Gazdar AF, Hsieh JT: Epigenetic regulation of a novel tumor suppressor gene (hDAB2IP) in prostate cancer cell lines. J Biol Chem 278: 3121-313 (2003).

Davis MM, Catino JJ, Satoh T, Kaziro Y, Perkins LM: Sequence of the cDNA encoding Ras GTPase-activating protein from rat. Gene 134: 305–306 (1993)

Dote H, Toyooka S, Tsukuda K, Yano M, Ouchida M, Doihara H, Suzuki M, Chen H, Hsieh JT, Gazdar AF, Shimizu N: Aberrant promoter methylation in human DAB2 interactive protein (*hDAB2IP*) gene in breast cancer. Clin Cancer Res 10: 2082-2089 (2004).

Foster BA, Gingrich JR, Kwon ED, Madias C, Greenberg NM: Characterization of prostatic epithelial cell lines derived from transgenic adenocarcinoma of the mouse prostate (TRAMP) model. Cancer Res 57: 3325-3330 (1997).

Fulop V, Colitti CV, Genest D, Berkowitz RS, Yiu GK, Ng SW, Szepesi J, Mok SC: DOC-2/hDAB2, a candidatetumor suppressor gene involved in the development of gestational trophoblastic diseases. Oncogene 17: 419–424 (1998).

Glanzer JG, Liao L, Baker T, McMullen MH, Langan AS, Crandall LZ, Vorce RL: Organization and regulation of the human rasGAP gene. Gene 285: 149-156 (2002).

Gingrich JR, Barrios RJ, Kattan MW, Nahm HS, Finegold MJ, Greenberg NM: Androgen-independent prostate cancer progression in the TRAMP model. Cancer Res 57: 4687-4691 (1997).

Greenberg NM, DeMayo F, Finegold MJ, Medina D, Tilley WD, Aspinall JO, Cunha GR, Donjacour AA, Matusik RJ, Rosen JM: Prostate cancer in a transgenic mouse. Proc Natl Acad Sci 92: 3439-3443 (1995).

Homayouni R, Magdaleno S, Keshvara L, Rice DS, Curran T: Interaction of Disabled-1 and the GTPase activating protein Dab2IP in mouse brain. Mol Brain Res 115: 121-129 (2003).

Jones PA, Takai D: Takai, The Role of DNA Methylation in Mammalian Epigenetics. Science 293: 1068-1070 (2001).

Kim JH, Liao D, Lau LF, Huganir R: SynGAP: a synaptic RasGAP that associates with the PSD-95/SAP90 protein family. Neuron 20: 683–691 (1998).

Li S, Satoh H, Watanabe T, Nakamura S, Hattori S: cDNA cloning and chromosomal mapping of a novel human GAP (GAP1M), a GTPase-activating protein of Ras. Genomics 35: 625–627 (1996).

Noto S, Maeda T, Hattori S, Inazawa J, Imamura M, Asaka M, Hatakeyama M: A novel human RasGAP-like gene that maps within the prostate cancer susceptibility locus at chromosome 1q25. FEBS Lett 441: 127–131 (1998).

Rebecchi MJ, Scarlata S: Pleckstrin homology domains: a common fold with diverse functions. Annu Rev Biophys Biomol Struct. 27: 503-528 (1998)

Shaw G: The pleckstrin homology domain: an intriguing multifunctional protein module. Bioessays 18: 35-46 (1996).

Tseng CP, Ely BD, Pong RC, Wang Z, Zhou J, Hsieh JT: The role of DOC-2/DAB2 protein phosphorylation in the inhibition of AP-1 activity. An underlying mechanism of its tumor-suppressive function in prostate cancer. J Biol Chem 274: 31981-31986 (1999).

von Bergh AR, Wijers PM, Groot AJ, van Zelderen-Bhola S, Falkenburg JH, Kluin PM, Schuuring E: Identification of a novel RAS GTPase-activating protein (*RASGAP*) gene at 9q34 as an *MLL* fusion partner in a patient with de novo acute myeloid leukemia. Genes Chromosomes Cancer 39: 324-334 (2004).

Wang Z, Tseng CP, Pong RC, Chen H, McConnell JD, Navone N, Hsieh JT: The mechanism of growth-inhibitory effect of DOC-2/DAB2 in prostate cancer. Characterization of a novel GTPase-activating protein associated with N-terminal domain of DOC-2/DAB2. J Biol Chem 277: 12622-12631 (2002).

Wolffe AP, Matzke MA: Epigenetics: Regulation through repression. Science 286: 481-486 (1999).

Zhang R, He X, Liu W, Lu M, Hsieh JT, Min W: AIP1 mediates TNF-α-induced ASK1 activation by facilitating dissociation of ASK1 from its inhibitor 14-3-3. J Clin Invest 111:1933-1943 (2003).

Zhou J, Hsieh JT: The inhibitory role of DOC-2/DAB2 in growth factor receptor-mediated signal cascade. DOC-2/DAB2-mediated inhibition of ERK phosphorylation via binding to Grb2. J Biol Chem 276: 27793–27798 (2001).

Zhou J, Scholes J, Hsieh JT: Characterization of a novel negative regulator (DOC-2/DAB2) of c-Src in normal prostatic epithelium and cancer. J Biol Chem 278: 6936-6941 (2003).

Figure Legends

Fig. 1. Structure of mDab2ip gene (A) Map of mDab2ip gene dispersed over an approximated 65kb. The exon 1 (black box) contains at least three variants (Ia, Ib, Ic), and the relative position of BAC clones is displayed. (B) Schematic display of two specific primers [Sp1 (outer) and Sp2 (inner)], and corresponding two universal primers for 5'RACE. (C) Alternative slicing of mDab2ip mRNA detected from brain, TRAMP-C1/TRAMP-C2, and kidney.

Fig. 2. Chromosomal localization of mDab2ip gene by FISH analysis (A) A DAPI stained chromosomes (blue) with hybridized probe (red) and (B) a reversed image of the chromosomal staining.

Fig. 3. The predicted functional domain of the mDab2ip protein (A) Alignment of mouse Dab2ip, human and rat DAB2IP proteins exhibited a high sequence homology. Potential functional domains are shaded and underlined. Shaded letters indicated the non-identical amino acids. (B) Schematic representation of mDab2ip protein. PH (peckstrin homology domain, amino acids 30-79); C2 (protein kinase C conserved region 2 domain, amino acids 90-189); RasGAP (Ras GTPase-activator domain, amino acids 212-539); PR (proline-rich domain, amino acids 796-805); and LZ (leucine zipper domain, amino acids 911-932)

Fig. 4. Expression profile of mDab2ip transcript in various mouse organs and TRAMP-C cell lines Real-time RT-PCR was performed to detect the expression level of mDab2ip mRNA from different mouse organs (A) and TRAMP-C lines (B). Mouse actin was used as an internal control. The fold induction was calculated as described in " Material and Method". To calculate the relative level of each sample, the lowest expression level of mDab2ip from Spleen (A), or TRAMP-C3 (B) was considered as 1. Variation of each sample is less than 10%.

Fig. 5. Characterization of mDab2ip gene promoters (A) The predicted regulatory sequences of the mDab2ip gene. Exon Ia sequence of the mDab2ip is *underlined.* The putative *cis*-acting elements are boxed. Primer sequences (light letters) and restriction endonucleases sites (light letters and underlined) were used in subsequent cloning for reporter constructs. The transcription start site (TSS) as +1 was predicted by 5' RACE. (B) Schematic representation of mDab2ip gene promoter region and its deletion mutants. A series of reporter gene constructs containing different fragment of mDab2ip promoter region was cloned in pGL-3 basic vector as described in "Materials and Methods". (C and D) Relative Luciferase activity (RLA) of the *mDAB2IP* promoter in TRAMP-C1 (C) and TRAMP-C3 (D). Bars, SD.

Table 1: PCR primers used on 5' RACE, Real-time RT-PCR, Probe and Promoter constructs

Application	Primer name	Primer 5'—3' sequence
5'RACE	Sp1 (outer)	ATACAGCACATCGTCCAGG
	Sp2 (inner)	GTTCTCCATCCACTTATCGCGC
Real-time RT-PCR	F-mDab2ip	CGATAAGTGGATGGAGAACCTGAG
	R-mDab2ip	AGATGCTGACGGTCTGGTAGCGTGC
	F-Actin	TGTGTGGATTGGTGGCTCTATC
	R-Actin	CTGCTTGCTGATCCACATCTG
Probe1 (800bp)	F1-*DAB2IP*	TCGTGGAAGGACTCATGACC
	R1-*DAB2IP*	TCCACCACCCTGTTGCTGTA
Probe 2 (300b)	F2-*DAB2IP*	TGGACGATGTGCTCTATGCC
	R2-*DAB2IP*	GGATGGTGATGGTTTGGTAG
Promoters	F1	GCTCCTCACCTGCCTCTTCATTAG
	F6	CCTGCTCTCCCAGCCTTAGTTTC
	F8	CACCAAGAGCCAGCCCAAAC
	F10	ACCCTTCGTTGCTTTCACCG
	F12	GAGTCCCTCGCTGTCCGATAC
	R2	TGCTCCTCCCCTCCAGATGTTC

Table 2: Different splicing variants of mouse Dab2ip

Exon	mDab2ip mRNA a (6109bp)	mDab2ip mRNA b (6392bp)	mDab2ip mRNA c (6051bp)	Amino acid (1065aa)
Ia	1-134			
Ib		1-417		
Ic			1-76	
II	135-288 (154) ***ATG (145-147)**	418-571 (154) *** ATG (428-440)**	77-230 (154) ***ATG (87-89)**	1-48 (48)
III	289-387 (99)	572-670 (99)	231-329(99)	49-81 (33)
IV	388-942 (555)	671-1225 (555)	330-884 (555)	82-266 (185)
V	943-1087 (145)	1226-1370 (145)	885-1029(145)	267-314 (48)
VI	1088-1232 (145)	1371-1515 (145)	1030-1174 (145)	315-363 (49)
VII	1233-1469 (237)	1516-1752 (237)	1175-1411 (237)	364-442 (79)
VIII	1470-1671 (202)	1753-1954 (202)	1412-1613(202)	443-509 (67)
IX	1672-1850 (179)	1955-2133 (179)	1614-1792179)	510-569(60)
X	1851-2739 (889)	2134-3022 (889)	1793-2681 (889)	570-865(296)
XI	2740-2892 (153)	3023-3175 (153)	2682-2834 (153)	866-915(50)
XII	2893-3086 (194)	3176-3369 (194)	2835-3028 (194)	916-981(66)
XIII	3087-3174 (88)	3370-3457 (88)	3029-3116(88)	982-1010(29)
XIV	3175-6109 (2935) ***TAA (3340-3342)**	3458-6392 (2935) ***TAA (3623-3625)**	3117-6051 (2935) ***TAA (3282-3284)**	1011-1065 (55)

Table 3: Exon-intron boundaries of the mDab2ip

Exon	Size (bp)	3' intron/5' exon boundary	3' exon/5' intron boundary	Intron	Size (kb)
Ia	134		TGAGAG/gtaggccc	1a	29.3
Ib	417	cctcctcag/GTCCCA	GCCAAG/gtctgtgac	1b	4.3
Ic	76	cctcctcag/GTCCCA	CACTTG/gtgagtggc	1c	10.0
II	154	cctcctcag/GTCCCA	TTCGAG/gtgggtgtc	2	1.0
III	99	gtttgacag/GTGACG	AACAAG/gtacctgta	3	0.9
IV	555	cttatgcag/GACAAC	GTGAAG/gtgagtgtg	4	2.7
V	145	cctccctag/GACTTT	CACTAG/gtagtgggg	5	0.1
VI	145	gcccacag/GTGAGT	CTACTG/gttagtgcca	6	2.7
VII	237	cctatgcag/TGCTTC	TGCCAA /gtgagtgtt	7	1.6
VIII	202	ttctgtccag/GTTTGG	GATCAG/gtgcctgtt	8	1.4
IX	179	attcttgtag/AGCGTT	CTCTGG /gtaagagc	9	0.9
X	889	tccctgcag/TCTGAT	AAGCAG/gtcagcacc	10	0.5
XI	153	ttcatcgtag/GGCCCT	GAAAAG/gtaaaactg	11	1.6
XII	194	tgctggcag/GATCTG	CAGCAG/gtgagcagg	12	4.2
XIII	88	ctgttcacag/GTTGAT	GCCCAG/gttggggctc	13	0.5
XIV	2935	gcccacag/GAAAAG			

A

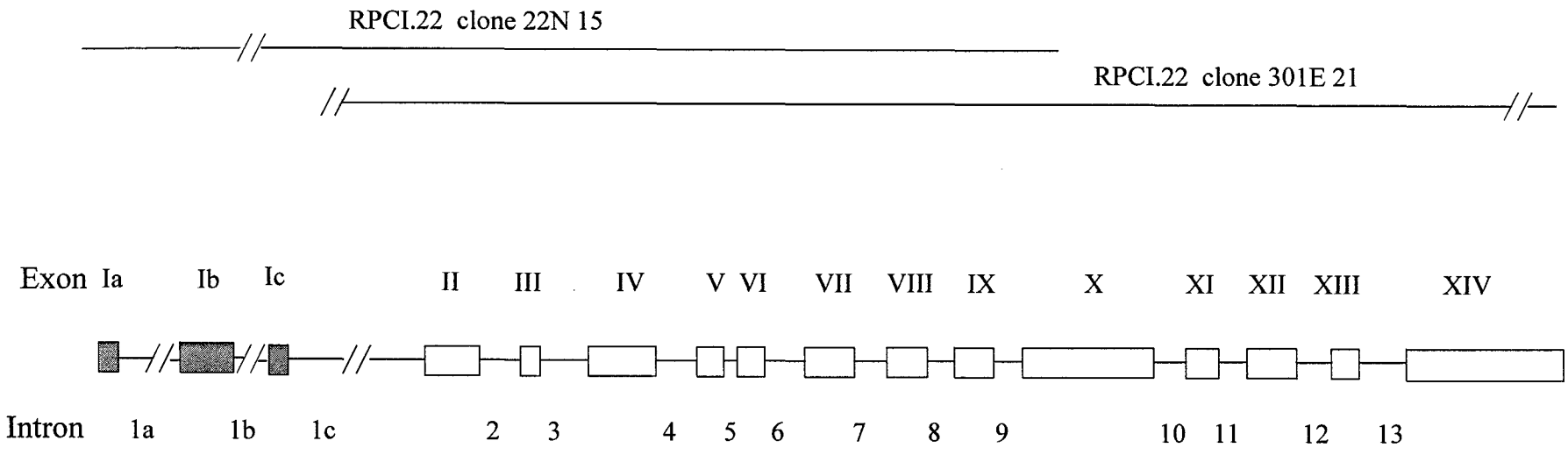
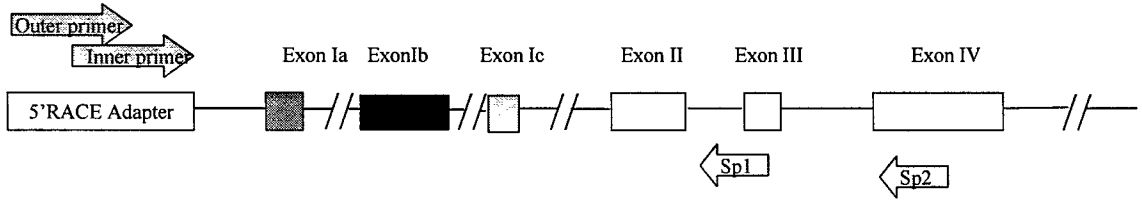

RPCI.22 clone 22N 15
RPCI.22 clone 301E 21
Exon Ia Ib Ic II III IV V VI VII VIII IX X XI XII XIII XIV
Intron 1a 1b 1c 2 3 4 5 6 7 8 9 10 11 12 13

Fig.1

B


Outer primer
Inner primer
Exon Ia
ExonIb
Exon Ic
Exon II
Exon III
Exon IV
5'RACE Adapter
Sp1
Sp2

C

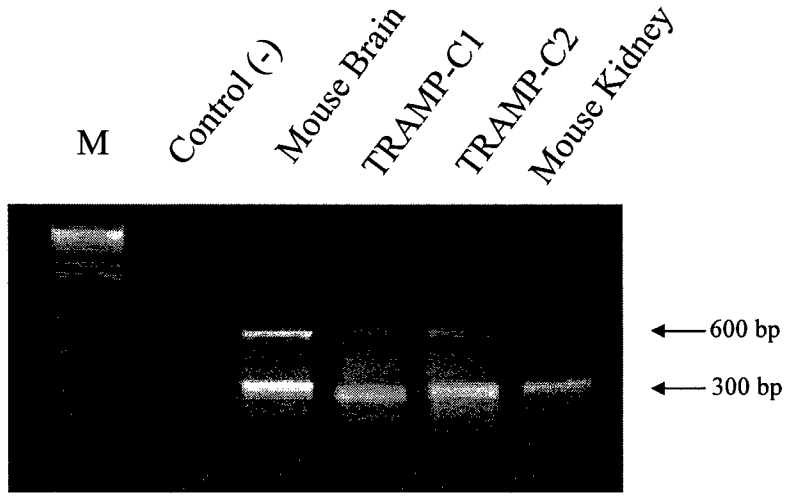
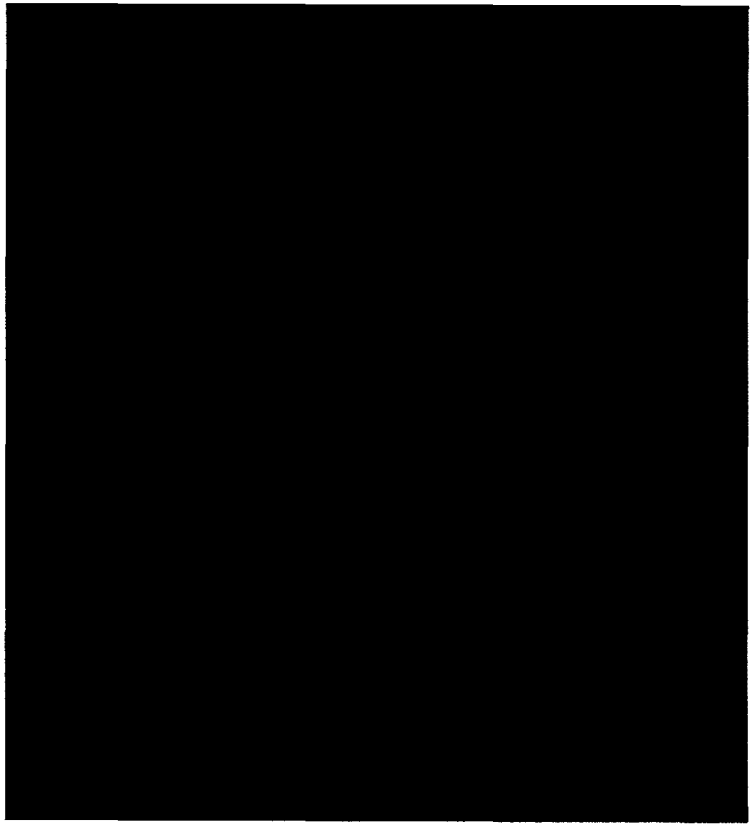

M
Control (-)
Mouse Brain
TRAMP-C1
TRAMP-C2
Mouse Kidney
600 bp
300 bp

Fig. 1

Figure 2A and B

A



B

Fig. 2

A

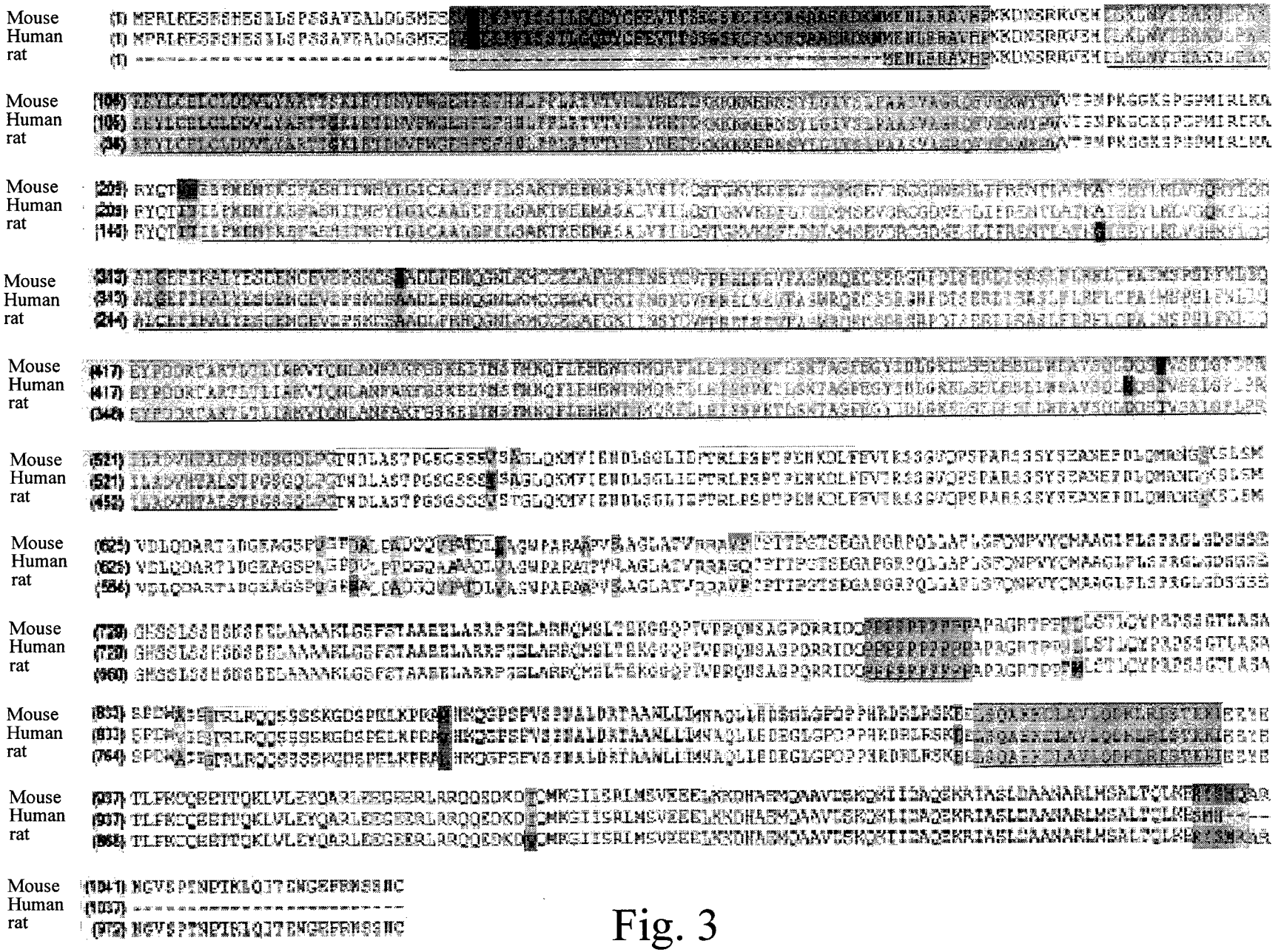


Fig. 3

B

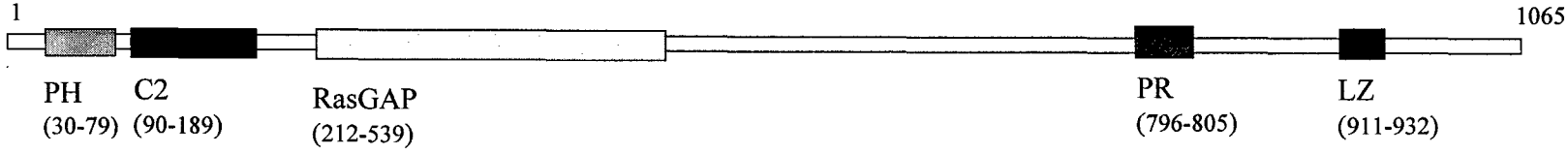


Fig.3

A

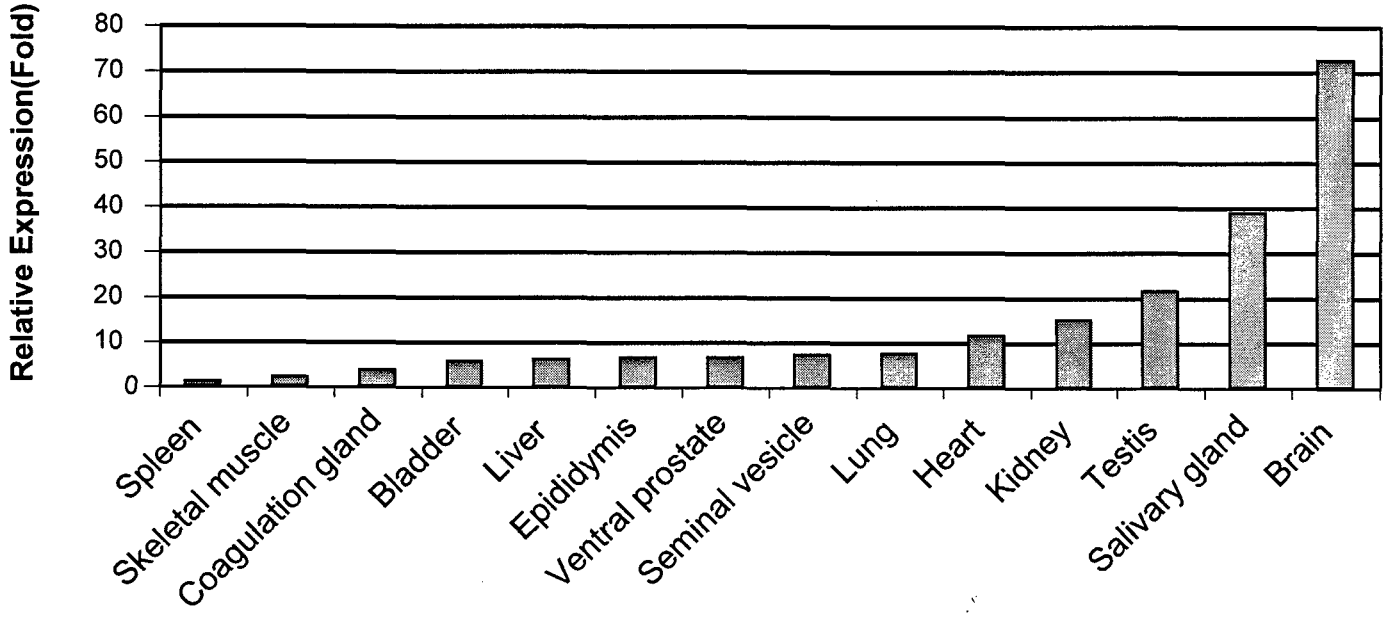

Relative Expression(Fold)
80
70
60
50
40
30
20
10
0
Spleen
Skeletal muscle
Coagulation gland
Bladder
Liver
Epididymis
Ventral prostate
Seminal vesicle
Lung
Heart
Kidney
Testis
Salivary gland
Brain

Fig. 4

B

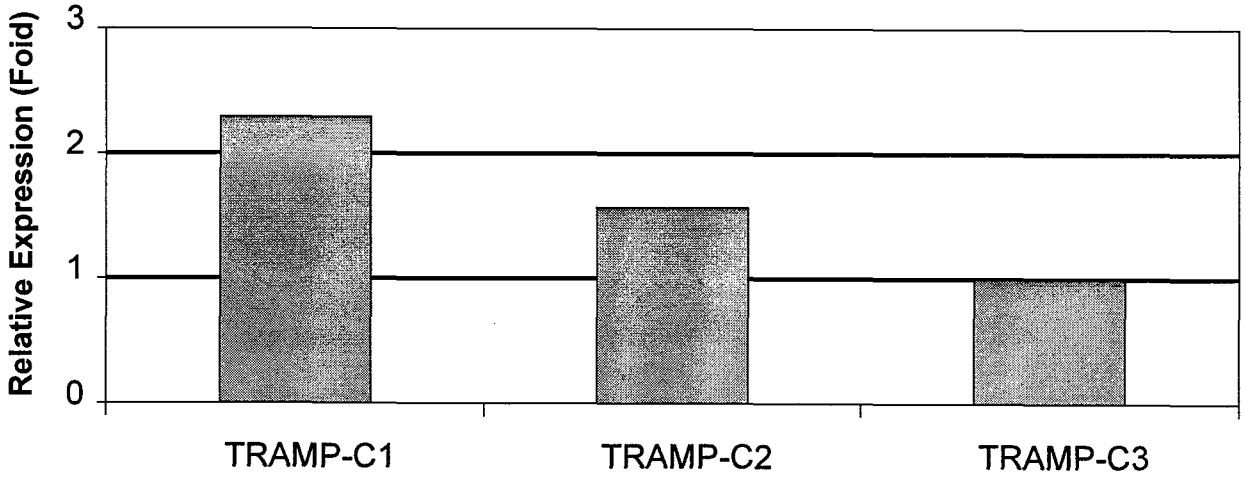


Fig. 4

A

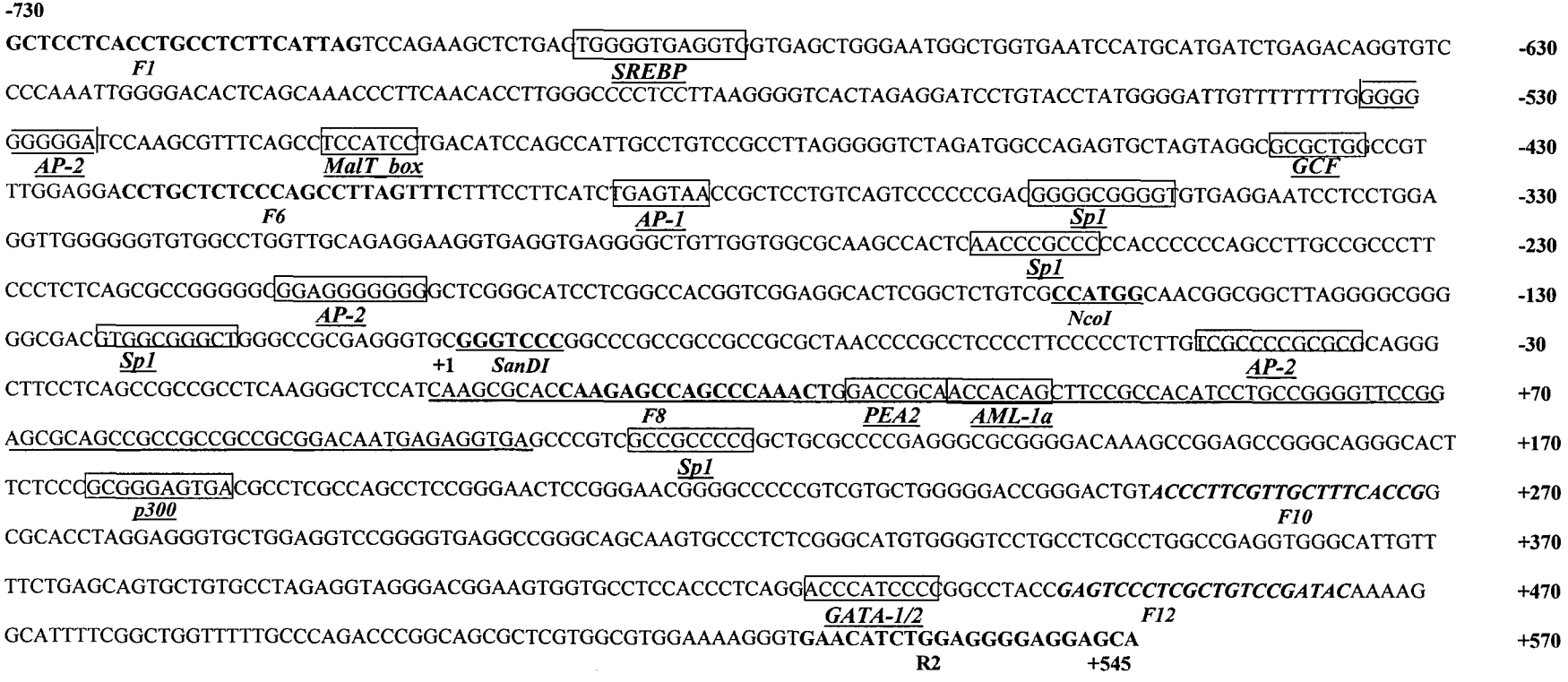

-730
GCTCCTCACCTGCCTCTTCATTAGTCCAGAAGCTCTGACTGGGGTGAGGTCGTGAGCTGGGAATGGCTGGTGAATCCATGCATGATCTGAGACAGGTGTC -630
F1
SREBP
CCCAAATTGGGGACACTCAGCAAACCCTTCAACACCTTGGGCCCCTCCTTAAGGGGTCACTAGAGGATCCTGTACCTATGGGGATTGTTTTTTTTGGGGG -530
GGGGGATCCAAGCGTTTCAGCCTCCATCCTGACATCCAGCCATTGCCTGTCCGCCTTAGGGGGTCTAGATGGCCAGAGTGCTAGTAGGCGCGCTGGCCGT -430
AP-2
MalT_box
GCF
TTGGAGGACCTGCTCTCCCAGCCTTAGTTTCTTTCCTTCATCTGAGTAACCGCTCCTGTCAGTCCCCCCGACGGGGCGGGGTGTGAGGAATCCTCCTGGA -330
F6
AP-1
Sp1
GGTTGGGGGGTGTGGCCTGGTTGCAGAGGAAGGTGAGGTGAGGGGCTGTTGGTGGCGCAAGCCACTCAACCCGCCCCCACCCCCCAGCCTTGCCGCCCTT -230
Sp1
CCCTCTCAGCGCCGGGGGCGGAGGGGGGGGCTCGGGCATCCTCGGCCACGGTCGGAGGCACTCGGCTCTGTCGCCATGGCAACGGCGGCTTAGGGGCGGG -130
AP-2
NcoI
GGCGACGTGGCGGGCTGGGCCGCGAGGGTGCGGGTCCCGGCCCGCCGCCGCCGCGCTAACCCCGCCTCCCCTTCCCCCTCTTGTCGCCCCGCGCGCAGGG -30
Sp1
+1
SanDI
AP-2
CTTCCTCAGCCGCCGCCTCAAGGGCTCCATCAAGCGCACCAAGAGCCAGCCCAAACTGGACCGCAACCACAGCTTCCGCCACATCCTGCCGGGGTTCCGG +70
F8
PEA2
AML-1a
AGCGCAGCCGCCGCCGCCGCGGACAATGAGAGGTGAGCCCGTCGCCGCCCCGCTGCGCCCCGAGGGCGCGGGGACAAAGCCGGAGCCGGGCAGGGCACT +170
Sp1
TCTCCCGCGGGAGTGACGCCTCGCCAGCCTCCGGGAACTCCGGGAACGGGGCCCCCGTCGTGCTGGGGGACCGGGACTGTACCCTTCGTTGCTTTCACCGG +270
p300
F10
CGCACCTAGGAGGGTGCTGGAGGTCCGGGGTGAGGCCGGGCAGCAAGTGCCCTCTCGGGCATGTGGGGTCCTGCCTCGCCTGGCCGAGGTGGGCATTGTT +370
TTCTGAGCAGTGCTGTGCCTAGAGGTAGGGACGGAAGTGGTGCCTCCACCCTCAGGACCCATCCCCGGCCTACCGAGTCCCTCGCTGTCCGATACAAAAG +470
GATA-1/2
F12
GCATTTTCGGCTGGTTTTTGCCCAGACCCGGCAGCGCTCGTGGCGTGGAAAAGGGTGAACATCTGGAGGGGAGGAGCA +570
R2
+545

Fig.5

B

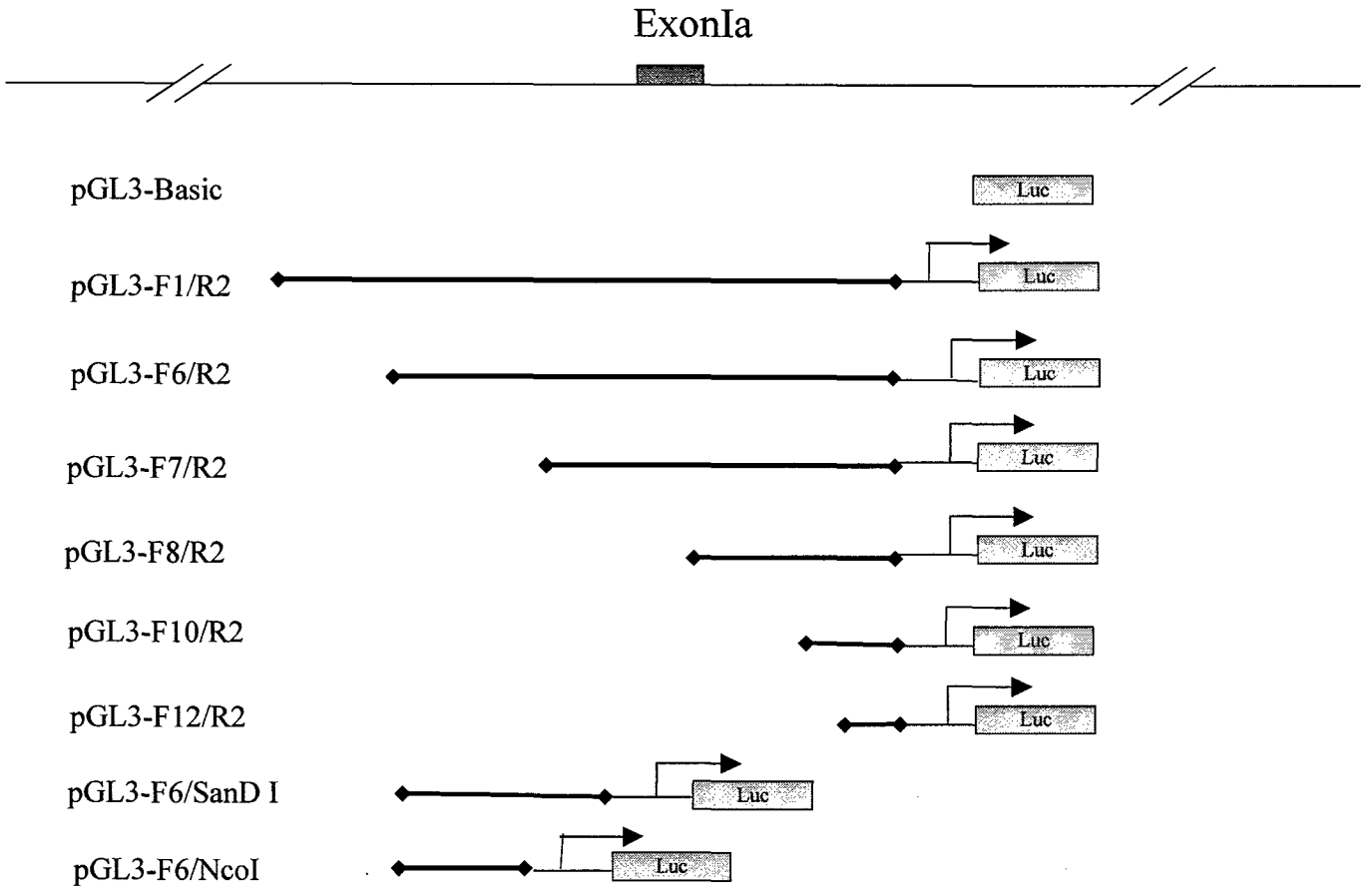

ExonIa
pGL3-Basic
Luc
pGL3-F1/R2
Luc
pGL3-F6/R2
Luc
pGL3-F7/R2
Luc
pGL3-F8/R2
Luc
pGL3-F10/R2
Luc
pGL3-F12/R2
Luc
pGL3-F6/SanD I
Luc
pGL3-F6/NcoI
Luc

Fig. 5

C

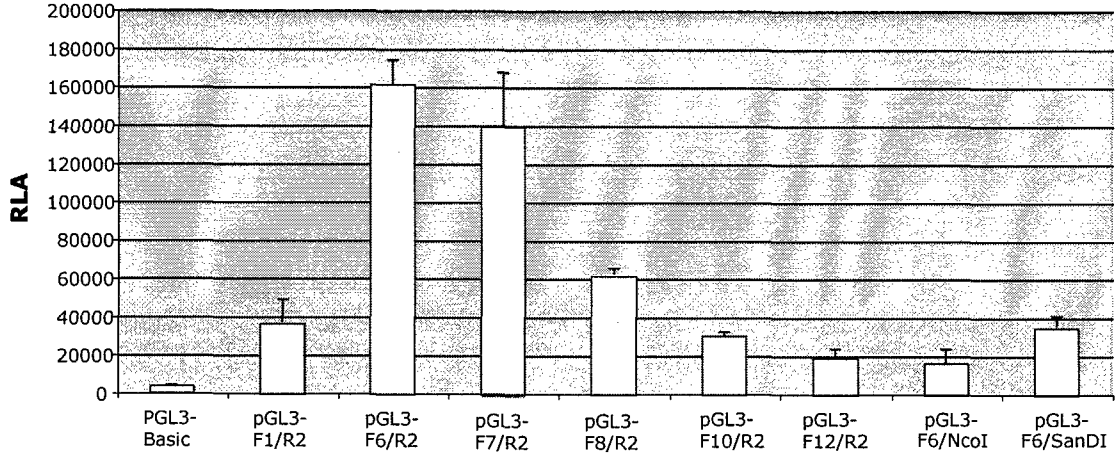

RLA
200000
180000
160000
140000
120000
100000
80000
60000
40000
20000
0
PGL3-Basic
pGL3-F1/R2
pGL3-F6/R2
pGL3-F7/R2
pGL3-F8/R2
pGL3-F10/R2
pGL3-F12/R2
pGL3-F6/NcoI
pGL3-F6/SanDI

Fig.5

D

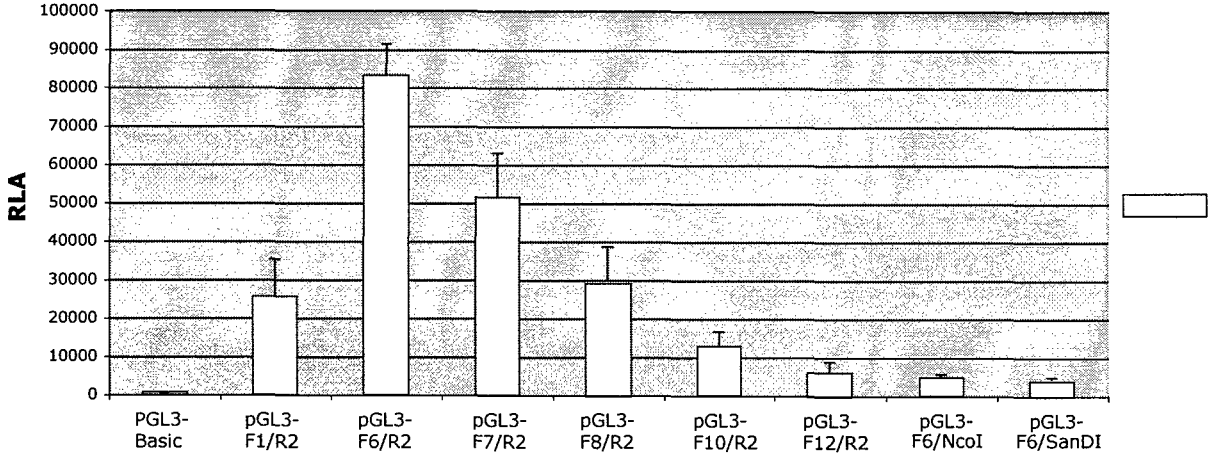

RLA
100000
90000
80000
70000
60000
50000
40000
30000
20000
10000
0
PGL3-Basic
pGL3-F1/R2
pGL3-F6/R2
pGL3-F7/R2
pGL3-F8/R2
pGL3-F10/R2
pGL3-F12/R2
pGL3-F6/NcoI
pGL3-F6/SanDI

Fig.5

9

EPIGENETICS IN PROSTATE CANCER

Jose A. Karam, Elie A. Benaim, Hong Chen,
Rey-Chen Pong and Jer-Tsong Hsieh

Department of Urology, UT Southwestern Medical Center
5323 Harry Hines Blvd., Dallas, TX, USA

Introduction

Prostate cancer (PCa) is the most common non-cutaneous cancer affecting males in the USA. One in six American men will be diagnosed with PCa in their lifetime. In 2004, a total of 230,110 new cases and 29,900 deaths are estimated to occur in the USA.[1] It has been well documented that alterations in genomic DNA, such as point mutations, homozygous deletions and loss of heterozygosity are linked to the pathogenesis of cancer,[2] including PCa.[3] However, the majority of studies have focused on the DNA sequence and to a lesser extent on DNA structure and its surrounding environment. Recently, investigators started looking at epigenetics as an alternative and complementary mechanism in the pathogenesis of cancer. The term "epigenetic" refers to the heritable changes in gene expression that are caused by mechanisms other than the alteration in the nucleotide sequence.[4] This concept generated tremendous new knowledge in understanding gene expression in mammalian cells.

The Role of CpG Dinucleotides in DNA Methylation

CpG dinucleotides can be either clustered as CpG islands, or dispersed. In the era before completion of the human genome project, a CpG island was defined as a stretch of 200-bp of DNA with a C + G content of 50% and

an observed CpG/expected CpG in excess of 0.6.[5] However, this definition included inactive and viral sequences within the human genome. When corrected for these "intrusive" sequences, a more biologically appropriate and more stringent definition of a CpG island would be a stretch of 500-bp of DNA with a C + G content of 55% and an observed CpG/expected CpG of more than 0.65.[6] Therefore, the definition of a CpG island is still arbitrary. More than half of the human genes are associated with unique CpG islands.[7] Reports from the human genome project have estimated that the human genome contains almost 30,000 CpG islands.[8]

CpG dinucleotides are present throughout the genome and are usually methylated when not associated with CpG islands. However, when the CpG dinucleotides are found in CpG islands, they are usually unmethylated, in particular if they are found in the promoter region of an active gene,[9] or methylated, when found in the 3′ end of the gene. However, CpG methylation the at 3′ end of the gene does not affect gene transcription.[10] In general, it is a rare event to find *de novo* CpG methylation in normal somatic cells. However, as cells age, especially when cultured *in vitro*, an increase in methylation has been documented.[11,12]

Cytosine methylation prevents gene expression by interfering with transcription initiation. DNA methylation has no effect on base pairing but can alter protein-DNA interaction by protruding into the major DNA groove.[13] Another explanation is that methylation results in decreased binding affinity of the transcription factors to gene promoter regions.[14]

Methyl Binding Proteins (MBP)

The first true MBP to be identified was MeCP2 (Methyl CpG-binding protein 2). It was found to specifically bind to the methyl group at position 5 of cytosine and to be active in somatic mammalian cells but not necessarily in embryonic stem cells.[15] This protein has been found most abundantly in the pericentromeric heterochromatin region[16] and its localization to this region has been shown to be dependent on the presence of methylated DNA.[17] MeCP2 specifically represses methylated promoters through a transcription repressor domain (TRD). MeCP2 can bind to DNA that contains one symmetrically methylated CpG pair and does not need chromatin disassembly in order to bind to DNA.[18] In fact, MeCP2 can displace histone H1 from

DNA and then exert its repression function, at least *in vitro*.[19] MeCP1 can also discriminate between methylated and unmethylated DNA, although needing a high amount of methylated CpG for its binding (more than 10 methylated CpGs).[20]

Four other methyl-binding domain (MBD)-containing proteins were identified by comparing the MBD domain of MeCP2 to Expressed Sequence Tags (EST) databases.[20] MBD1-mediated activity is Histone Deacetylase (HDAC)-dependent, as its actions are partially inhibited by HDAC inhibitors (HDIs). However, the identity of this HDAC is still unknown.[21] MBD1 interacts with a histone H3 methyltransferase as well as heterochromatin protein 1 (a methyl-lysine binding protein) resulting in chromatin compaction and transcription repression.[22] In the prostate, MBD1 is highly expressed in benign prostate hyperplasia and low grade PCa. However, MBD1 expression decreases in high grade PCas.[23] MBD2, which is a part of the MeCP1 complex, has transcription repression activity[24] as well as DNA demethylase activity.[25,26] MBD3 is similar in structure to MBD2, however no demethylase activity has been demonstrated.[27] MBD4 has DNA glycosylase activity, therefore effectively removing uracil or thymidine from a mismatched CpG location, at least *in vitro*. Binding experiments showed that mutations at methyl CpG sites can be reduced by the activity of MBD4.[28]

DNA Methyltransferases (DNMTs)

The conversion of cytosine (C) into 5-methylcytosine (5mC) is catalyzed by enzymes termed DNA methyl transferases (DNMTs), using S-adenosylmethionine (SAM) as a methyl donor.[29] Three active DNMTs have been characterized. DNMT1 is responsible for methylating daughter DNA strands during the S-phase, through its interaction with Proliferating Cell Nuclear Antigen (PCNA).[30] DNMT1 is involved in maintaining the methylation status of genes and this task is possible due to its high affinity to hemi-methylated DNA.[31] When DNMT1 is absent in mice, DNA methylation is severely affected, resulting in an embryonic lethal phenotype.[32] DNMT3a and DNMT3b have some structural similarities, having the N-terminal as the regulatory subunit and the C-terminal as the catalytic subunit. In contrast to DNMT1, both DNMT3a and DNMT3b can methylate unmethylated and hemi-methylated DNA.[33]

An elegant study in *Drosophila* showed that DNMT3a, but not DNMT1, acts as a *de novo* methyltransferase.[34] Therefore, DNMT3a establishes the gene methylation pattern and then DNMT1 maintains this pattern in the future generations. When DNMT3a or DNMT3b were knocked out, the resulting mice died at 4 weeks of life or during embryonic development, respectively.[35] Increased DNMT mRNA has been documented in colon and lung cancer.[36–39] However, DNA hypermethylation is not necessarily due to overexpression of DNMT.[40]

DNMT Inhibitors

DNMT inhibitors act mainly by inhibiting methylation; however, other mechanisms are involved. After phosphorylation, these compounds incorporate into DNA or RNA.[41] After incorporating into DNA, they covalently bind DNMT resulting in its inhibition[42–44] without causing DNA demethylation *per se.*[44] Additionally, their incorporation into DNA causes structural instability resulting in DNA damage.[45]

Currently, five DNMT inhibitors have been used in preclinical/clinical trials, namely 5-azacytidine (azacytidine), 5-aza-2′-deoxycytidine (decitabine), dihydro-5-azacytidine (DHAC), arabinofuranosyl-5-azacytosine (fazarabine)[46] and most recently zebularine. Zebularine is the only DNMT inhibitor that can be given orally, while the other DNMT inhibitors are given parenterally.[47]

DNMT inhibitors have been used in preclinical studies with very good tumor control rate. However, in clinical trials, the results are not so dramatic, especially with solid tumors, where limited efficacy has been encountered.[46] Decitabine has been used in phase II trial in patients with hormone-independent metastatic prostate cancer, however, the results were not encouraging.[48] Most of the DNMT inhibitors have a relatively high cytotoxicity profile and can only demethylate genes as long as they are present in the cell surroundings, therefore limiting their potential clinical usefulness, as they cannot be administered for prolonged periods of time. However, a recent *in vitro* study showed that zebularine can suppress DNMT1 expression, demethylate p16 and restore p16 expression for up to 40 days in continuously treated T24 bladder cancer cells, without causing pronounced cytotoxicity. In addition, when zebularine is given at a lower

dose following an initial dose of decitabine, a profound demethylation of p16 is observed. These results suggest the usefulness of combining an initial bolus of a parenteral DNMT inhibitor followed by a maintenance dose of the oral agent zebularine.[49] However, proof of their usefulness in a preclinical and ultimately clinical setting is yet to be established.

Recently, RNA interference (RNAi) has been used to knock out DNMT1,[50] which composes the majority of DNMT activity in cancer cells,[51] resulting in reactivation of repressed genes. Another study, however, showed that both DNMT1 and DNMT3b need to be shut down to re-express silenced genes.[52] These results imply the need to treat cancer cells with compounds that can affect more than one type of DNMT, as cancer cells might be able to overcome the deficiency in one DNMT.[53] Two phase I clinical trials using MG98, an antisense oligodeoxynucleotide against DNMT1, have been recently conducted to study the MG98 safety profile and dosage regimens in patients with cancer.[54,55]

Histones

In mammalian cells, genomic DNA is always associated with histone proteins to form chromatin structure. The basic unit of chromatin is the nucleosome, which is composed of 146 base pairs of DNA that are wrapped twice around a disk-like complex made of 8 histone proteins (2 of each of histones H2A, H2B, H3, and H4). A stable nucleosome core is assembled by H3 and H4 heterodimerization, followed by H3 dimerization resulting in a $(H3\text{-}H4)_2$ tetramer.[56] Subsequently, H2A and H2B heterodimerize and each dimer attaches to one side of the $(H3\text{-}H4)_2$ tetramer.[56] This yields an octamer of histones on which DNA can be wrapped.[57] Adjacent nucleosomes are brought together by histone H1. The N- and C-termini of histone H1 are able to bind to DNA within the nucleosome in addition to the "linker" DNA between the nucleosomes. This binding and subsequent neutralization of the acidic DNA results in the formation of higher order chromatin.[58–60] The amino acids at the N-terminal of the histone protrude out of the nucleosome, thereby allowing for specific reversible modifications and interaction with surrounding factors. This N-terminal is rich in the basic (positively charged) amino acids like lysine and serine, which allows for reversible histone modification by methylation, acetylation,

phosphorylation, ubiquitination and most recently sumoylation. When this positively charged N-terminal of the amino acid is free, it results in tight binding of histones to DNA phosphates, which are negatively charged, therefore inhibiting access of transcription factors to the gene promoter region and subsequently silencing gene expression.

Acetylation of histones is mediated by histone acetyltransferases (HATs) using acetyl CoA as the acetyl moiety donor, resulting in activation of gene transcription. HATs do not directly bind to DNA, instead, they are subunits in coactivator complexes that mediate transcriptional activity recovery. Examples of such coactivator HATs in humans are p300/CBP[61] and TAFII250,[62] which is a component of human RNA polymerase II, an enzyme involved in transcription. Steroid Receptor Coactivator-1 (SRC-1)[63] and ACTR[64] are also coactivators with HAT activity that interact with nuclear receptors in a hormone-dependent fashion. pCAF[65] has the ability to associate with p300/CBP,[66] ACTR[64] and SRC-1,[63] resulting in a multitude of HATs in the same complex. Acetylation of histones has been linked with transcriptional activation.

The second histone modification is phosphorylation of the Serine-10 of histone H3. This modification has been implicated in both activation of transcription[67] and chromosomal condensation in mitosis. This phosphorylation corresponds to the activation of early-immediate genes, such as c-Fos[68] and has been shown to occur through the Rsk-2,[69] Msk-1[70] and Snf-1 kinases.[71] Interestingly, the latter histone kinase works in concert with HAT to activate gene transcription.[71,72] Also, I kappa B kinase α (IKKα) has been reported to phosphorylate histone H3, subsequently allowing for specific H3 acetylation through CREB-binding protein (CBP), resulting in activation of nuclear factor kappa B (NF-κB) responsive genes.[73] It is not clear how histone phosphorylation activates gene transcription; however, it could be related to the fact that adding the negatively charged phosphates to histone N-termini disrupts the histone-DNA interaction, resulting in increased access of transcription factors to promoter regions.[74]

The third histone modification is due to methylation of either arginine or lysine residues. Arginine methylation at histone H3 and H4 is mediated by the histone methyltransferase (HMT) CARM1[75,76] and PRMT1,[77] respectively, through transfer of a methyl group from SAM. The end product

results in gene activation. Histone lysine methylation, on the other hand, can result in either gene transcription activation or repression. Suvar3-9 has HMT activity, specifically methylating Lysine-9 of histone H3 (K9-H3),[78] subsequently attracting the heterochromatin protein HP1.[79,80] resulting in heterochromatin assembly[81] and gene silencing. Human HMTs at K9-H3 include SUV39H1[82] (causing retinoblastoma (Rb)-mediated transcription repression) and EZH2 (a member of the polycomb group of transcriptional repressors).[83,84] In addition to methylation at K9-H3, EZH2 has the ability to methylate K27-H3, resulting also in transcription repression.[85] EZH2 expression was reported to be increased in metastatic prostate cancer and in localized prostate cancer. In the latter case, it indicates a poor patient prognosis and outcome.[85] Interestingly, both monomethylation[86] and trimethylation[87] at K9-H3 can control and trigger DNA methylation *per se.* Recently, it has been shown that methylation levels of K9-H3 differ depending on their location in the chromosome and that different HMTs are responsible for their methylation at each of these sites: monomethylated and dimethylated K9-H3 are localized in silenced euchromatin regions and their methylation is mediated by G9a HMT, while trimethylated K9-H3 was abundant in pericentromeric regions, with SUV39H1 and SUV39H2 responsible for their methylation.[88] In contrast, methylation at Lysine-4 of histone H3 (K4-H3) by SET7/Set9.[89,90] results in transcription activation. In LNCaP prostate cancer cells, the activated androgen receptor (AR) binds to the androgen responsive element (ARE) of the prostate specific antigen (PSA) gene, resulting in decreased methylation of K4-H3 of the PSA enhancer and promoter and increased PSA transcription.[91]

The fourth covalent histone modification is ubiquitination.[92] Ubiquitin is a 76-amino acid peptide that can attach to the C-terminus of histone H2A and likely to that of H2B as well. Ubiquitination of histone H2A coincides with transcriptional activity.[93] However, ubiquitination of histone H2B results in histone H3 methylation, thereby causing transcription repression.[94]

Recently, histone sumoylation has been implicated in transcriptional repression of gene activity. SUMO (small ubiquitin-related modifier) is involved in post-translational modification of several proteins and it is mediated by the same enzymatic cascade that catalyzes ubiquitination. Sumoylation of histone H4 results in recruitment of HDAC and the

heterochromatin protein HP1, therefore causing transcriptional repression.[95] Collectively, these histone modifications may constitute a "Histone code" that is able to specify patterns of gene expression.[96]

Histone Deacetylases (HDACs)

In mammals, HDACs have been grouped into 3 classes. Class I HDACs (HDAC 1, 2, 3, 8) have catalytic site homology at their C-termini and class II HDACs (HDAC 4, 5, 6, 7, 9,10) share homology at their catalytic C-termini and regulatory N-termini.[97] Class III is the conserved NAD-dependent Sir2 group of deacetylases.[97] Interestingly, class I and II, but not class III HDACs, are inhibited by Trichostatin A (TSA) and suberoylanilide hydroxamic acid (SAHA). Similar to HATs, there is evidence that HDACs do not directly bind to DNA. Instead, they are part of the transcription regulator complexes.[98] HDACs do not show complete redundancy in their functions. Class I HDACs are almost exclusively located in the nucleus. On the other hand, class II HDACs translocate between the cytoplasm and nucleus.[97,99]

Histone Deacetylase Inhibitors (HDIs)

HDIs can be classified on the basis of their different structures into hydroxamates (TSA and SAHA), cyclic tetrapeptides (apidicin depsipeptide and depudecin), carboxylates (sodium phenylbutyrate and valproic acid) and benzamides (CI-994 and MS-27-275).[100–108]

The inhibition of HDACs, as mediated by HDIs via interacting with a zinc active site in the HDAC moiety,[109] could result in induction of differentiation, growth arrest and/or apoptosis.[110] These effects are attributed to a subset of genes whose expression has been altered, or returned back to normal upon exposure to HDIs and histone hyperacetylation,[111] as seen in single-gene,[112] as well as high-throughput studies.[113]

Several HDIs, including SAHA, phenylbutyrate and depsipeptide have been used in Phase I and Phase II clinical trials.[114] SAHA can be administered orally or intravenously and has been shown to increase the levels of acetylated histones in peripheral blood mononuclear cells and reduce tumor activity in pretreated patients with hematologic and solid

cancers.[115] SAHA was also effective in increasing the intratumor acetylated histone levels and inhibiting the growth of prostate cancer xenografts in nude mice.[116]

The Interaction Between DNA Methylation and Histone Modifications in Gene Regulation

Recently, an interaction between histone modifications and DNA methylation has been discovered and has been intensively studied. Jones and colleagues described a system where methylated cytosine attracts the methyl-binding protein MeCP2, which in turn binds a HDAC-corepressor complex, therefore resulting in transcription inhibition.[117] In addition, DNA methylation and histone methylation also interact, as MeCP2 can recruit a HMT, resulting in methylation of K9-H3.[118] More recently, it has been shown that DNMT1 and DNMT3a interact with SUV39H1 (a K9-H3 HMT) as well as HP1, providing more evidence for the close association between DNA and histone methylation.[119]

A question that has been recently addressed is whether methylation of DNA precedes histone modifications or *vice versa.*[120,121] Most of the currently available data supports the dominant role of methylation in the epigenetic control of promoter activation, as DNMT inhibitors are more important than HDAC inhibitors in initiating gene reactivation.[122] In these cases, even if HDAC inhibitors cause histone acetylation, methyl binding proteins such as MeCP2 are still bound to methylated cytosine, thereby needing a DNMT inhibitor to release the methylation and subsequently disengaging from MeCP2.[123] However, recent data showed that methylation of K9-H3 and subsequent gene silencing can occur prior to methylation of CpG in the gene promoter.[53] Both phenomena are present to some extent and the relative importance of one or the other could be dependent on specific tumor tissue, cell type, experimental conditions and the specific gene under study.

The fact that DNA methylation and histone deacetylation cooperate to silence genes has been exploited to develop new therapeutic regimens. DNA microarray analysis[113] experiments show that the use of DNMT inhibitors and HDAC inhibitors results in a synergistic activation of specific tumor suppressor genes,[122,124,125] as well as affecting global gene

expression. In addition, low doses of DNMT inhibitors and HDIs are combined in order to decrease the occurrence of side effects and cause a synergistic inhibition of tumor growth.[126] Decitabine and phenylbutyrate was found to have a synergistic effect in preventing the formation of lung tumors in mice.[127]

Epigenetics in the Pathogenesis and Diagnosis of PCa

Patra and colleagues have demonstrated that elevated DNMT and HDAC expression is associated with PCa, indicating that epigenetic regulation plays an important role in PCa pathogenesis.[128] Several genes have been described as being methylated in PCa and only to a limited extent or not at all in normal prostates. Some genes are methylated in early stages of PCa, potentially making them useful as diagnostic markers. Other genes are methylated in advanced stages of PCa and can be used as prognostic markers.

GSTP1

The most commonly studied epigenetically-controlled gene in PCa is the Glutathione-S-transferase-π1 (GSTP1) gene. GSTP1 is responsible for intracellular detoxification reactions by conjugating free radicals to glutathione.[129,130] The first evidence of GSTP1 methylation in PCa was demonstrated by Lee and colleagues,[131] when they described that GSTP1 was not expressed in several PCa cell lines due to promoter hypermethylation.

The revolution in detection of methylated DNA came in 1996 when Herman and colleagues described methylation-specific PCR (MSP), which can offer higher sensitivity and specificity in detecting minute amounts of methylated DNA.[132] Recently, Harden and colleagues employed a quantitative MSP (qMSP) assay for GSTP1 in conjunction with routine prostate biopsy histology in order to improve the PCa detection. They found that histology alone detected 39/61 tumors (64% sensitivity), while GSTP1 qMSP with histology detected 48/61 tumors (79% sensitivity) with a 100% specificity. However, when the threshold of qMSP is decreased, 4/11 samples that were labeled as normal on histology were positive on qMSP. This raises the question that such patients should be monitored more intensively, as

preneoplastic lesions can harbor GSTP1 methylation. However, more controlled trials are needed to justify such a follow up.[133]

By combining laser capture microdissection and MSP, recent data demonstrated the absence of GSTP-1 methylation in patients with normal or benign hyperplastic prostates, while GSTP1 was methylated in 6.3% with prostatic proliferative inflammatory atrophy (PIA), 68.8% with high-grade prostatic intraepithelial neoplasia (HGPIN) and 90.9% with PCa. These results suggest a progressive increase in GSTP1 methylation during prostate carcinogenesis and PIA as a potential precursor of HGPIN/PCa based on the presence of GSTP1 methylation.[134]

MSP has also been used to detect GSTP1 methylation from several sources with good success, including blood, bone marrow, urine, ejaculate, lymph nodes (LNs) after pelvic LN dissection, prostate biopsies, TURP and prostatectomy specimens (Table 1). MSP was able to detect GSTP1 methylation in all of these tissues, with a low rate of false positive results.

Table 1. GSTP1 Detection in Human Tissue/Body Fluids using MSP

Sample Origin	Patient Diagnosis	% Methylation	Reference No.(s)
Prostate			
Biopsy	Cancer	89	133
Biopsy washings	Cancer	100	180
	PIN	67	
	BPH	0	
Prostate resection	Cancer	88	134
	HGPIN	69	
	PIA	6	
	BPH	0	
	Normal	0	
Pelvic lymph nodes	Cancer	90	181
	Normal	11	
Bone marrow	Cancer	40	181
Urine	Cancer	27–39	182, 183
	Normal	0–3	
Plasma	Cancer	36–72	184, 185
	Normal	0	
Ejaculate	Cancer	50	184
	Normal	0	

Detection of cancer in LN allows for a more accurate assessment of metastatic disease and better adjuvant therapy. Detection of methylated GSTP1 in blood, urine and ejaculate potentially allows for earlier detection of PCa. In addition, its presence in blood, urine, or bone marrow could prove to be a useful prognostic marker, surrogate marker for predicting cancer volume during therapy and a means of follow up after definitive therapy.

Membrane Receptors

CD44

CD44 is an integral membrane glycoprotein involved in cell to cell adhesion and cell-extracellular matrix interaction.[135] It has been shown that CD44 promoter hypermethylation inversely correlates with the expression level of CD44 in PCa cell lines.[136] The methylation status of CD44 was examined in prostatectomy specimens, which revealed that 77.5% of PCa specimens harbored CD44 methylation, in contrast to only 10% of the matched normal controls, suggesting the possible involvement of CD44 methylation and transcriptional repression in the prostate carcinogenesis.[137] Additionally, the hypermethylation of CD44 has been found to be more common in advanced stages of PCa.[138] Recently, Woodson and colleagues reported that hypermethylation of CD44 was 1.7 times more common in black patients than in white patients with PCa.[139] Ekici and colleagues studied CD44 expression in a cohort of patients with PCa and reported that the presence of CD44 is significantly higher in non-metastatic versus metastatic groups, correlates inversely with pathologic stage and disease progression and positively with PSA-free survival.[140]

CAR

Coxsackie and adenovirus receptor (CAR), first identified as the high affinity receptor for both coxsackie and adenovirus (type 2 and 5)[141,142] is a typical immunoglobulin (Ig)-like membrane protein with two Ig domains that may have adhesion activity.[143] Like many other cell adhesion molecules, the loss of CAR is often detected in several cancer types.[144–149] In PCa, decreased expression of CAR is found in primary tumors[146] and increased expression of CAR can reduce tumor growth of

PCa *in vitro* and *in vivo.*[150] Data from our laboratory indicate that the CpG islands in the CAR promoter are unmethylated; however, the decreased expression of CAR is due to histone deacetylation at the CAR promoter.[151] In PCa, the use of HDIs increases the expression of CAR, which further enhances the adenovirus susceptibility of PCa cells.[151] Thus, combining HDIs with recombinant adenovirus could lead to a more effective treatment regimen for PCa patients.

Nuclear Receptors

AR

The AR has been shown to be down-regulated as a result of its promoter methylation.[152,153] AR is methylated in only 8% of PCas and in none of the normal prostates. The PCas with methylated AR were exclusively high stage, indicating that AR methylation is a relatively late event in prostate carcinogenesis.[154]

Estrogen Receptor (ER)

When studied in clinical specimens, ER methylation was detected in 60% of BPH, 80% of low-stage PCa and 100% of high-stage PCa specimens. The difference of ER methylation was highly significant in PCa compared to BPH.[155] Another study investigated the different subtypes of ERs in paired PCa samples and discovered that ERα-A and ERα-B gene promoters were methylated in 98% and 92% of PCa samples, respectively, while they were unmethylated in normal prostate samples. ERα-C was not methylated in any PCa samples. In addition, ERβ was methylated in 78% of PCas, with no methylation in normal prostates.[154] Such differences in ER gene methylation could imply a different role of each subtype of ER in the pathogenesis of PCa.

Retinoid Acid Receptor-β2 (RARβ2)

RARβ2 is a nuclear receptor that binds to the retinoic acid responsive element (RARE) found in retinoic acid pathway genes and other transcription factor genes.[156] The hypermethylation of RARβ2 has been

reported in 53%–83% of PCas, compared to 0%–3% in normal tissues/BPH.[157–159] It has been shown that decitabine and TSA can induce RARβ2 gene expression, indicating that histone deacetylation and DNA methylation are responsible for silencing of the RARβ2 gene.[157]

Ras Effector Proteins

In PCa, enhanced expression of Ras protein correlates with increased tumor grade.[160] Several studies indicated that most metastatic tumors expressed Ras protein, while only a fifth of primary tumors did.[160–162] Surprisingly, PCa cells demonstrate an extremely low rate of mutation in the *Ras* gene.[162–164] This implies that other effectors regulating Ras activity may be involved in increasing Ras protein levels in PCa.

RASSF1A

RASSF1A, a Ras effector homologue, is a tumor suppressor gene that is down-regulated due to its promoter hypermethylation in several cancers[165,166]including PCa.[158,167] *RASSF1A* hypermethylation was found in 53%–71% of PCa specimens and it was more prevalent in patients with Gleason scores 7–10.[158,167] These results suggest that *RASSF1A* gene methylation may reflect an aggressive PCa phenotype and could potentially be used as a marker in this context.

Human Disabled-2 Interacting Protein (hDAB2IP)

hDAB2IP is a new member of Ras-GTPase activating protein family that maintains Ras inactivation status.[168] The down-regulation of hDAB2IP is often associated with many androgen-independent PCa cell lines and increased expression of hDAB2IP can suppress the growth of PCa.[168] Analysis of hDAB2IP gene promoter reveals that it is hypermethylated in PCa cell lines but not in normal epithelial cells. Both DNMT inhibitors and HDIs can induce the expression level of hDAB2IP,[169,170] indicating that the epigenetic machinery plays an important role in modulating hDAB2IP expression during PCa progression.

Nuclear Proteins

Cyclin D2

Cyclins are proteins involved in cell cycle regulation by interacting with cyclin-dependent kinases.[171] Cyclin D2 is involved in the transition from G1 to S phase during mitosis.[172] Padar and colleagues found that PCas are more commonly methylated (32%) than normal prostate tissue (6%) independent of age. In addition, methylation was significantly higher in patients with Gleason scores >7.[173] This finding suggests that loss of cyclin D2 could be involved in PCa progression.

ZNF185

ZNF185, or Zinc Finger 185 is a protein that belongs to the LIM domain protein family.[174] LIM proteins play a role in cellular growth and differentiation.[175] In a recent study, ZNF185 was found to be down-regulated in a genomic screening of PCas. After treatment of PCa cell lines with a DNMT inhibitor, ZNF185 expression was reestablished. Using MSP, ZNF185 hypermethylation was detected in PCa, but not in any normal prostate samples. When stratified into different stages, ZNF185 is methylated in 36.3%, 50% and 100% of PCa samples with Gleason 6, Gleason 9 and from metastatic sites, respectively. These data indicate a role for ZNF185 promoter hypermethylation in the progression of PCa and its potential application as a tumor marker for PCa.[176]

Bone Morphogenic Protein-6 (BMP-6)

BMP-6 belongs to the transforming growth factor-β superfamily, which is involved in the formation of bone and cartilage.[177] In contrast to the above genes, where their expression has been silenced by promoter hypermethylation, BMP-6 gene methylation is decreased in PCa. This loss of BMP-6 promoter methylation and subsequent gene activation leads to overexpression of BMP-6 protein in both primary and secondary sites of PCa with advanced metastasis.[178] BMP-6 expression in PCa is

associated with a decreased bone-metastasis-free survival as compared to those with no BMP-6 expression.[179] The re-expression of BMP-6 by aggressive PCa could be one way through which these cancers metastasize to bone.

Conclusion

In addition to the genetic mutations of tumor suppressor genes associated with PCa, recent data clearly indicate that epigenetic alterations are also involved in this silencing. Unlike genetic changes, epigenetic modifications are potentially reversible, which can open a new avenue of cancer therapy; several inhibitors of DNMT and HDAC are currently being studied in clinical trials for several cancers. Even so, more controlled studies on epigenetics in PCa are needed to develop new methods for early cancer detection, to predict patient prognosis and to ultimately treat patients with PCa.

References

1. Jemal A, Tiwari RC, Murray T, *et al.* (2004) Cancer statistics, 2004. *CA Cancer J Clin* 54:8–29.
2. Knudson AG, Jr (1985) Hereditary cancer, oncogenes, and antioncogenes. *Cancer Res* 45:1437–1443.
3. Ozen M, Pathak S (2000) Genetic alterations in human prostate cancer: a review of current literature. *Anticancer Res* 20:1905–1912.
4. Bird A (2002) DNA methylation patterns and epigenetic memory. *Genes Dev* 16:6–21.
5. Gardiner-Garden M, Frommer M (1987) CpG islands in vertebrate genomes. *J Mol Biol* 196:261–282.
6. Takai D, Jones PA (2002) Comprehensive analysis of CpG islands in human chromosomes 21 and 22. *Proc Natl Acad Sci USA* 99:3740–3745.
7. Antequera F, Bird A (1993) Number of CpG islands and genes in human and mouse. *Proc Natl Acad Sci USA* 90:11995–11999.
8. Lander ES, Linton LM, Birren B, *et al.* (2001) Initial sequencing and analysis of the human genome. *Nature* 409:860–921.
9. Bird AP (1986) CpG-rich islands and the function of DNA methylation. *Nature* 321:209–213.

10. Larsen F, Solheim J, Prydz H (1993) A methylated CpG island 3′ in the apolipoprotein-E gene does not repress its transcription. *Hum Mol Genet* 2:775–780.
11. Jones PA, Wolkowicz MJ, Rideout WM, 3rd, *et al.* (1990) *De novo* methylation of the MyoD1 CpG island during the establishment of immortal cell lines. *Proc Natl Acad Sci USA* 87:6117–6121.
12. Antequera F, Boyes J, Bird A (1990) High levels of *de novo* methylation and altered chromatin structure at CpG islands in cell lines. *Cell* 62:503–514.
13. Razin A, Riggs AD (1980) DNA methylation and gene function. *Science* 210:604–610.
14. Clark SJ, Harrison J, Molloy PL (1997) Sp1 binding is inhibited by (m)Cp(m)CpG methylation. *Gene* 195:67–71.
15. Meehan RR, Lewis JD, Bird AP (1992) Characterization of MeCP2, a vertebrate DNA binding protein with affinity for methylated DNA. *Nucleic Acids Res* 20:5085–5092.
16. Lewis JD, Meehan RR, Henzel WJ, *et al.* (1992) Purification, sequence, and cellular localization of a novel chromosomal protein that binds to methylated DNA. *Cell* 69:905–914.
17. Nan X, Tate P, Li E, Bird A (1996) DNA methylation specifies chromosomal localization of MeCP2. *Mol Cell Biol* 16:414–421.
18. Ballestar E, Wolffe AP (2001) Methyl-CpG-binding proteins. Targeting specific gene repression. *Eur J Biochem* 268:1–6.
19. Nan X, Campoy FJ, Bird A (1997) MeCP2 is a transcriptional repressor with abundant binding sites in genomic chromatin. *Cell* 88:471–481.
20. Hendrich B, Bird A (1998) Identification and characterization of a family of mammalian methyl-CpG binding proteins. *Mol Cell Biol* 18:6538–6547.
21. Ng HH, Jeppesen P, Bird A (2000) Active repression of methylated genes by the chromosomal protein MBD1. *Mol Cell Biol* 20:1394–1406.
22. Fujita N, Watanabe S, Ichimura T, *et al.* (2003) Methyl-CpG binding domain 1 (MBD1) interacts with the Suv39h1-HP1 heterochromatic complex for DNA methylation-based transcriptional repression. *J Biol Chem* 278:24132–24138.
23. Patra SK, Patra A, Zhao H, Carroll P, Dahiya R (2003) Methyl-CpG-DNA binding proteins in human prostate cancer: expression of CXXC sequence containing MBD1 and repression of MBD2 and MeCP2. *Biochem Biophys Res Commun* 302:759–766.
24. Ng HH, Zhang Y, Hendrich B, *et al.* (1999) MBD2 is a transcriptional repressor belonging to the MeCP1 histone deacetylase complex. *Nat Genet* 23: 58–61.

25. Bhattacharya SK, Ramchandani S, Cervoni N, Szyf M (1999) A mammalian protein with specific demethylase activity for mCpG DNA. *Nature* 397:579–583.
26. Detich N, Theberge J, Szyf M (2002) Promoter-specific activation and demethylation by MBD2/demethylase. *J Biol Chem* 277:35791–35794.
27. Wade PA, Gegonne A, Jones PL, Ballestar E, Aubry F, Wolffe AP (1999) Mi-2 complex couples DNA methylation to chromatin remodelling and histone deacetylation. *Nat Genet* 23:62–66.
28. Hendrich B, Hardeland U, Ng HH, Jiricny J, Bird A (1999) The thymine glycosylase MBD4 can bind to the product of deamination at methylated CpG sites. *Nature* 401:301–304.
29. Sano H, Sager R (1980) Deoxyribonucleic acid methyltransferase from the eukaryote, *Chlamydomonas reinhardi. Eur J Biochem* 105:471–480.
30. Chuang LS, Ian HI, Koh TW, Ng HH, Xu G, Li BF (1997) Human DNA-(cytosine-5) methyltransferase-PCNA complex as a target for p21WAF1. *Science* 277:1996–2000.
31. Pradhan S, Bacolla A, Wells RD, Roberts RJ (1999) Recombinant human DNA (cytosine-5) methyltransferase. I. Expression, purification, and comparison of *de novo* and maintenance methylation. *J Biol Chem* 274: 33002–33010.
32. Li E, Bestor TH, Jaenisch R (1992) Targeted mutation of the DNA methyltransferase gene results in embryonic lethality. *Cell* 69:915–926.
33. Okano M, Xie S, Li E (1998) Cloning and characterization of a family of novel mammalian DNA (cytosine-5) methyltransferases. *Nat Genet* 19: 219–220.
34. Lyko F, Ramsahoye BH, Kashevsky H, *et al.* (1999) Mammalian (cytosine-5) methyltransferases cause genomic DNA methylation and lethality in *Drosophila. Nat Genet* 23:363–366.
35. Okano M, Bell DW, Haber DA, Li E (1999) DNA methyltransferases Dnmt3a and Dnmt3b are essential for *de novo* methylation and mammalian development. *Cell* 99:247–257.
36. Issa JP, Vertino PM, Wu J, *et al.* (1993) Increased cytosine DNA-methyltransferase activity during colon cancer progression. *J Natl Cancer Inst* 85:1235–1240.
37. Robertson KD, Uzvolgyi E, Liang G, *et al.* (1999) The human DNA methyltransferases (DNMTs) 1, 3a and 3b: coordinate mRNA expression in normal tissues and overexpression in tumors. *Nucleic Acids Res* 27: 2291–2298.

38. Belinsky SA, Nikula KJ, Baylin SB, Issa JP (1996) Increased cytosine DNA-methyltransferase activity is target-cell-specific and an early event in lung cancer. *Proc Natl Acad Sci USA* 93:4045–4050.
39. Melki JR, Warnecke P, Vincent PC, Clark SJ (1998) Increased DNA methyltransferase expression in leukaemia. *Leukemia* 12:311–316.
40. Eads CA, Danenberg KD, Kawakami K, Saltz LB, Danenberg PV, Laird PW (1999) CpG island hypermethylation in human colorectal tumors is not associated with DNA methyltransferase overexpression. *Cancer Res* 59: 2302–2306.
41. Lubbert M (2000) DNA methylation inhibitors in the treatment of leukemias, myelodysplastic syndromes and hemoglobinopathies: clinical results and possible mechanisms of action. *Curr Top Microbiol Immunol* 249:135–164.
42. Bouchard J, Momparler RL (1983) Incorporation of 5-Aza-2′-deoxycytidine-5′-triphosphate into DNA. Interactions with mammalian DNA polymerase alpha and DNA methylase. *Mol Pharmacol* 24:109–114.
43. Santi DV, Garrett CE, Barr PJ (1983) On the mechanism of inhibition of DNA-cytosine methyltransferases by cytosine analogs. *Cell* 33:9–10.
44. Juttermann R, Li E, Jaenisch R (1994) Toxicity of 5-aza-2′-deoxycytidine to mammalian cells is mediated primarily by covalent trapping of DNA methyltransferase rather than DNA demethylation. *Proc Natl Acad Sci USA* 91: 11797–11801.
45. Lin KT, Momparler RL, Rivard GE (1981) High-performance liquid chromatographic analysis of chemical stability of 5-aza-2′-deoxycytidine. *J Pharm Sci* 70:1228–1232.
46. Goffin J, Eisenhauer E (2002) DNA methyltransferase inhibitors-state of the art. *Ann Oncol* 13:1699–1716.
47. Cheng JC, Matsen CB, Gonzales FA, *et al.* (2003) Inhibition of DNA methylation and reactivation of silenced genes by zebularine. *J Natl Cancer Inst* 95:399–409.
48. Thibault A, Figg WD, Bergan RC, *et al.* (1998) A phase II study of 5-aza-2′deoxycytidine (decitabine) in hormone independent metastatic (D2) prostate cancer. *Tumori* 84:87–89.
49. Cheng JC, Weisenberger DJ, Gonzales FA, *et al.* (2004) Continuous zebularine treatment effectively sustains demethylation in human bladder cancer cells. *Mol Cell Biol* 24:1270–1278.
50. Robert MF, Morin S, Beaulieu N, *et al.* (2003) DNMT1 is required to maintain CpG methylation and aberrant gene silencing in human cancer cells. *Nat Genet* 33:61–65.

51. Rhee I, Jair KW, Yen RW, *et al.* (2000) CpG methylation is maintained in human cancer cells lacking DNMT1. *Nature* 404:1003–1007.
52. Rhee I, Bachman KE, Park BH, *et al.* (2002) DNMT1 and DNMT3b cooperate to silence genes in human cancer cells. *Nature* 416:552–556.
53. Bachman KE, Park BH, Rhee I, *et al.* (2003) Histone modifications and silencing prior to DNA methylation of a tumor suppressor gene. *Cancer Cell* 3:89–95.
54. Davis AJ, Gelmon KA, Siu LL, *et al.* (2003) Phase I and pharmacologic study of the human DNA methyltransferase antisense oligodeoxynucleotide MG98 given as a 21-day continuous infusion every 4 weeks. *Invest New Drugs* 21:85–97.
55. Stewart DJ, Donehower RC, Eisenhauer EA, *et al.* (2003) A phase I pharmacokinetic and pharmacodynamic study of the DNA methyltransferase 1 inhibitor MG98 administered twice weekly. *Ann Oncol* 14:766–774.
56. Eickbush TH, Moudrianakis EN (1978) The histone core complex: an octamer assembled by two sets of protein-protein interactions. *Biochemistry* 17:4955–4964.
57. Luger K, Mader AW, Richmond RK, Sargent DF, Richmond TJ (1997) Crystal structure of the nucleosome core particle at 2.8 A resolution. *Nature* 389:251–260.
58. Allan J, Mitchell T, Harborne N, Bohm L, Crane-Robinson C (1986) Roles of H1 domains in determining higher order chromatin structure and H1 location. *J Mol Biol* 187:591–601.
59. Carruthers LM, Bednar J, Woodcock CL, Hansen JC (1998) Linker histones stabilize the intrinsic salt-dependent folding of nucleosomal arrays: mechanistic ramifications for higher-order chromatin folding. *Biochemistry* 37:14776–14787.
60. Howe L, Iskandar M, Ausio J (1998) Folding of chromatin in the presence of heterogeneous histone H1 binding to nucleosomes. *J Biol Chem* 273: 11625–11629.
61. Ogryzko VV, Schiltz RL, Russanova V, Howard BH, Nakatani Y (1996) The transcriptional coactivators p300 and CBP are histone acetyltransferases. *Cell* 87:953–959.
62. Mizzen CA, Yang XJ, Kokubo T, *et al.* (1996) The TAF(II)250 subunit of TFIID has histone acetyltransferase activity. *Cell* 87:1261.
63. Spencer TE, Jenster G, Burcin MM, *et al.* (1997) Steroid receptor coactivator-1 is a histone acetyltransferase. *Nature* 389:194–198.
64. Chen H, Lin RJ, Schiltz RL, *et al.* (1997) Nuclear receptor coactivator ACTR is a novel histone acetyltransferase and forms a multimeric activation complex with P/CAF and CBP/p300. *Cell* 90:569–580.

65. Yang WM, Inouye C, Zeng Y, Bearss D, Seto E (1996) Transcriptional repression by YY1 is mediated by interaction with a mammalian homolog of the yeast global regulator RPD3. *Proc Natl Acad Sci USA* 93: 12845–12850.
66. Yang XJ, Ogryzko VV, Nishikawa J, Howard BH, Nakatani Y (1996) A p300/CBP-associated factor that competes with the adenoviral oncoprotein E1A. *Nature* 382:319–324.
67. Nowak SJ, Corces VG (2000) Phosphorylation of histone H3 correlates with transcriptionally active loci. *Genes Dev* 14:3003–3013.
68. Mahadevan LC, Willis AC, Barratt MJ (1991) Rapid histone H3 phosphorylation in response to growth factors, phorbol esters, okadaic acid, and protein synthesis inhibitors. *Cell* 65:775–783.
69. Sassone-Corsi P, Mizzen CA, Cheung P, *et al.* (1999) Requirement of Rsk-2 for epidermal growth factor-activated phosphorylation of histone H3. *Science* 285:886–891.
70. Thomson S, Clayton AL, Hazzalin CA, Rose S, Barratt MJ, Mahadevan LC (1999) The nucleosomal response associated with immediate-early gene induction is mediated via alternative MAP kinase cascades: MSK1 as a potential histone H3/HMG-14 kinase. *EMBO J* 18:4779–4793.
71. Lo WS, Duggan L, Tolga NC, *et al.* (2001) Snf1-a histone kinase that works in concert with the histone acetyltransferase Gcn5 to regulate transcription. *Science* 293:1142–1146.
72. Cheung P, Tanner KG, Cheung WL, Sassone-Corsi P, Denu JM, Allis CD (2000) Synergistic coupling of histone H3 phosphorylation and acetylation in response to epidermal growth factor stimulation. *Mol Cell* 5: 905–915.
73. Yamamoto Y, Verma UN, Prajapati S, Kwak YT, Gaynor RB (2003) Histone H3 phosphorylation by IKK-alpha is critical for cytokine-induced gene expression. *Nature* 423:655–659.
74. Cheung P, Allis CD, Sassone-Corsi P (2000) Signaling to chromatin through histone modifications. *Cell* 103:263–271.
75. Schurter BT, Koh SS, Chen D, *et al.* (2001) Methylation of histone H3 by coactivator-associated arginine methyltransferase 1. *Biochemistry* 40: 5747–5756.
76. Chen D, Ma H, Hong H, *et al.* (1999) Regulation of transcription by a protein methyltransferase. *Science* 284:2174–2177.
77. Wang H, Huang ZQ, Xia L, *et al.* (2001) Methylation of histone H4 at arginine 3 facilitating transcriptional activation by nuclear hormone receptor. *Science* 293:853–857.

78. Rea S, Eisenhaber F, O'Carroll D, *et al.* (2000) Regulation of chromatin structure by site-specific histone H3 methyltransferases. *Nature* 406:593–599.
79. Bannister AJ, Zegerman P, Partridge JF, *et al.* (2001) Selective recognition of methylated lysine 9 on histone H3 by the HP1 chromo domain. *Nature* 410:120–124.
80. Lachner M, O'Carroll D, Rea S, Mechtler K, Jenuwein T (2001) Methylation of histone H3 lysine 9 creates a binding site for HP1 proteins. *Nature* 410:116–120.
81. Nakayama J, Rice JC, Strahl BD, Allis CD, Grewal SI (2001) Role of histone H3 lysine 9 methylation in epigenetic control of heterochromatin assembly. *Science* 292:110–113.
82. Nielsen SJ, Schneider R, Bauer UM, *et al.* (2001) Rb targets histone H3 methylation and HP1 to promoters. *Nature* 412:561–565.
83. Kuzmichev A, Nishioka K, Erdjument-Bromage H, Tempst P, Reinberg D (2002) Histone methyltransferase activity associated with a human multiprotein complex containing the Enhancer of Zeste protein. *Genes Dev* 16: 2893–2905.
84. Czermin B, Melfi R, McCabe D, Seitz V, Imhof A, Pirrotta V (2002) *Drosophila* enhancer of Zeste/ESC complexes have a histone H3 methyltransferase activity that marks chromosomal Polycomb sites. *Cell* 111:185–196.
85. Varambally S, Dhanasekaran SM, Zhou M, *et al.* (2002) The polycomb group protein EZH2 is involved in progression of prostate cancer. *Nature* 419:624–629.
86. Tamaru H, Selker EU (2001) A histone H3 methyltransferase controls DNA methylation in *Neurospora crassa. Nature* 414:277–283.
87. Tamaru H, Zhang X, McMillen D, *et al.* (2003) Trimethylated lysine 9 of histone H3 is a mark for DNA methylation in *Neurospora crassa. Nat Genet* 34:75–79.
88. Rice JC, Briggs SD, Ueberheide B, *et al.* (2003) Histone methyltransferases direct different degrees of methylation to define distinct chromatin domains. *Mol Cell* 12:1591–1598.
89. Wang H, Cao R, Xia L, *et al.* (2001) Purification and functional characterization of a histone H3-lysine 4-specific methyltransferase. *Mol Cell* 8: 1207–1217.
90. Nishioka K, Chuikov S, Sarma K, *et al.* (2002) Set9, a novel histone H3 methyltransferase that facilitates transcription by precluding histone tail modifications required for heterochromatin formation. *Genes Dev* 16:479–489.
91. Kim J, Jia L, Tilley WD, Coetzee GA (2003) Dynamic methylation of histone H3 at lysine 4 in transcriptional regulation by the androgen receptor. *Nucleic Acids Res* 31:6741–6747.

92. Goldknopf IL, Taylor CW, Baum RM, *et al.* (1975) Isolation and characterization of protein A24, a "histone-like" non-histone chromosomal protein. *J Biol Chem* 250:7182–7187.
93. Pham AD, Sauer F (2000) Ubiquitin-activating/conjugating activity of TAFII250, a mediator of activation of gene expression in *Drosophila*. *Science* 289:2357–2360.
94. Sun ZW, Allis CD (2002) Ubiquitination of histone H2B regulates H3 methylation and gene silencing in yeast. *Nature* 418:104–108.
95. Shiio Y, Eisenman RN (2003) Histone sumoylation is associated with transcriptional repression. *Proc Natl Acad Sci USA* 100:13225–13230.
96. Turner BM (2002) Cellular memory and the histone code. *Cell* 111: 285–291.
97. Grozinger CM, Chao ED, Blackwell HE, Moazed D, Schreiber SL (2001) Identification of a class of small molecule inhibitors of the sirtuin family of NAD-dependent deacetylases by phenotypic screening. *J Biol Chem* 276: 38837–38843.
98. Khochbin S, Verdel A, Lemercier C, Seigneurin-Berny D (2001) Functional significance of histone deacetylase diversity. *Curr Opin Genet Dev* 11: 162–166.
99. de Ruijter AJ, van Gennip AH, Caron HN, Kemp S, van Kuilenburg AB (2003) Histone deacetylases (HDACs): characterization of the classical HDAC family. *Biochem J* 370:737–749.
100. Yoshida M, Kijima M, Akita M, Beppu T (1990) Potent and specific inhibition of mammalian histone deacetylase both *in vivo* and *in vitro* by trichostatin A. *J Biol Chem* 265:17174–17179.
101. Richon VM, Emiliani S, Verdin E, *et al.* (1998) A class of hybrid polar inducers of transformed cell differentiation inhibits histone deacetylases. *Proc Natl Acad Sci USA* 95:3003–3007.
102. Singh SB, Zink DL, Liesch JM, *et al.* (2002) Structure and chemistry of apicidins, a class of novel cyclic tetrapeptides without a terminal alpha-keto epoxide as inhibitors of histone deacetylase with potent antiprotozoal activities. *J Org Chem* 67:815–825.
103. Furumai R, Matsuyama A, Kobashi N, *et al.* (2002) FK228 (depsipeptide) as a natural prodrug that inhibits class I histone deacetylases. *Cancer Res* 62:4916–4921.
104. Kwon HJ, Owa T, Hassig CA, Shimada J, Schreiber SL (1998) Depudecin induces morphological reversion of transformed fibroblasts via the inhibition of histone deacetylase. *Proc Natl Acad Sci USA* 95:3356–3361.

105. Boivin AJ, Momparler LF, Hurtubise A, Momparler RL (2002) Antineoplastic action of 5-aza-2′-deoxycytidine and phenylbutyrate on human lung carcinoma cells. *Anticancer Drugs* 13:869–874.
106. Phiel CJ, Zhang F, Huang EY, Guenther MG, Lazar MA, Klein PS (2001) Histone deacetylase is a direct target of valproic acid, a potent anticonvulsant, mood stabilizer, and teratogen. *J Biol Chem* 276:36734–36741.
107. Prakash S, Foster BJ, Meyer M, *et al.* (2001) Chronic oral administration of CI-994: a phase 1 study. *Invest New Drugs* 19:1–11.
108. Saito A, Yamashita T, Mariko Y, *et al.* (1999) A synthetic inhibitor of histone deacetylase, MS-27-275, with marked *in vivo* antitumor activity against human tumors. *Proc Natl Acad Sci USA* 96:4592–4597.
109. Finnin MS, Donigian JR, Cohen A, *et al.* (1999) Structures of a histone deacetylase homologue bound to the TSA and SAHA inhibitors. *Nature* 401:188–193.
110. Marks P, Rifkind RA, Richon VM, Breslow R, Miller T, Kelly WK (2001) Histone deacetylases and cancer: causes and therapies. *Nat Rev Cancer* 1:194–202.
111. Van Lint C, Emiliani S, Verdin E (1996) The expression of a small fraction of cellular genes is changed in response to histone hyperacetylation. *Gene Expr* 5:245–253.
112. Butler LM, Zhou X, Xu WS, *et al.* (2002) The histone deacetylase inhibitor SAHA arrests cancer cell growth, up-regulates thioredoxin-binding protein-2, and down-regulates thioredoxin. *Proc Natl Acad Sci USA* 99:11700–11705.
113. Suzuki H, Gabrielson E, Chen W, *et al.* (2002) A genomic screen for genes upregulated by demethylation and histone deacetylase inhibition in human colorectal cancer. *Nat Genet* 31:141–149.
114. Marks PA, Miller T, Richon VM (2003) Histone deacetylases. *Curr Opin Pharmacol* 3:344–351.
115. Kelly WK, Richon VM, O'Connor O, *et al.* (2003) Phase I clinical trial of histone deacetylase inhibitor: suberoylanilide hydroxamic acid administered intravenously. *Clin Cancer Res* 9:3578–3588.
116. Butler LM, Agus DB, Scher HI, *et al.* (2000) Suberoylanilide hydroxamic acid, an inhibitor of histone deacetylase, suppresses the growth of prostate cancer cells *in vitro* and *in vivo*. *Cancer Res* 60:5165–5170.
117. Jones PL, Veenstra GJ, Wade PA, *et al.* (1998) Methylated DNA and MeCP2 recruit histone deacetylase to repress transcription. *Nat Genet* 19:187–191.

118. Fuks F, Hurd PJ, Wolf D, Nan X, Bird AP, Kouzarides T (2003) The methyl-CpG-binding protein MeCP2 links DNA methylation to histone methylation. *J Biol Chem* 278:4035–4040.
119. Fuks F, Hurd PJ, Deplus R, Kouzarides T (2003) The DNA methyltransferases associate with HP1 and the SUV39H1 histone methyltransferase. *Nucleic Acids Res* 31:2305–2312.
120. Jones PA, Baylin SB (2002) The fundamental role of epigenetic events in cancer. *Nat Rev Genet* 3:415–428.
121. Li E (2002) Chromatin modification and epigenetic reprogramming in mammalian development. *Nat Rev Genet* 3:662–673.
122. Cameron EE, Bachman KE, Myohanen S, Herman JG, Baylin SB (1999) Synergy of demethylation and histone deacetylase inhibition in the re-expression of genes silenced in cancer. *Nat Genet* 21:103–107.
123. El-Osta A, Kantharidis P, Zalcberg JR, Wolffe AP (2002) Precipitous release of methyl-CpG binding protein 2 and histone deacetylase 1 from the methylated human multidrug resistance gene (MDR1) on activation. *Mol Cell Biol* 22:1844–1857.
124. Shaker S, Bernstein M, Momparler LF, Momparler RL (2003) Preclinical evaluation of antineoplastic activity of inhibitors of DNA methylation (5-aza-2′-deoxycytidine) and histone deacetylation (trichostatin A, depsipeptide) in combination against myeloid leukemic cells. *Leuk Res* 27:437–444.
125. Gagnon J, Shaker S, Primeau M, Hurtubise A, Momparler RL (2003) Interaction of 5-aza-2′-deoxycytidine and depsipeptide on antineoplastic activity and activation of 14-3-3sigma, E-cadherin and tissue inhibitor of metalloproteinase 3 expression in human breast carcinoma cells. *Anticancer Drugs* 14:193–202.
126. Zhu WG, Otterson GA (2003) The interaction of histone deacetylase inhibitors and DNA methyltransferase inhibitors in the treatment of human cancer cells. *Curr Med Chem Anti-Canc Agents* 3:187–199.
127. Belinsky SA, Klinge DM, Stidley CA, *et al.* (2003) Inhibition of DNA methylation and histone deacetylation prevents murine lung cancer. *Cancer Res* 63:7089–7093.
128. Patra SK, Patra A, Dahiya R (2001) Histone deacetylase and DNA methyltransferase in human prostate cancer. *Biochem Biophys Res Commun* 287:705–713.
129. Coles B, Ketterer B (1990) The role of glutathione and glutathione transferases in chemical carcinogenesis. *Crit Rev Biochem Mol Biol* 25:47–70.

130. Rushmore TH, Pickett CB (1993) Glutathione S-transferases, structure, regulation, and therapeutic implications. *J Biol Chem* 268:11475–11478.
131. Lee WH, Morton RA, Epstein JI, *et al.* (1994) Cytidine methylation of regulatory sequences near the pi-class glutathione S-transferase gene accompanies human prostatic carcinogenesis. *Proc Natl Acad Sci USA* 91: 11733–11737.
132. Herman JG, Graff JR, Myohanen S, Nelkin BD, Baylin SB (1996) Methylation-specific PCR: a novel PCR assay for methylation status of CpG islands. *Proc Natl Acad Sci USA* 93:9821–9826.
133. Harden SV, Sanderson H, Goodman SN, *et al.* (2003) Quantitative GSTP1 methylation and the detection of prostate adenocarcinoma in sextant biopsies. *J Natl Cancer Inst* 95:1634–1637.
134. Nakayama M, Bennett CJ, Hicks JL, *et al.* (2003) Hypermethylation of the human glutathione S-transferase-pi gene (GSTP1) CpG island is present in a subset of proliferative inflammatory atrophy lesions but not in normal or hyperplastic epithelium of the prostate: a detailed study using laser-capture microdissection. *Am J Pathol* 163:923–933.
135. Naot D, Sionov RV, Ish-Shalom D (1997) CD44: structure, function, and association with the malignant process. *Adv Cancer Res* 71:241–319.
136. Verkaik NS, Trapman J, Romijn JC, van der Kwast TH, van Steenbrugge GJ (1999) Down-regulation of CD44 expression in human prostatic carcinoma cell lines is correlated with DNA hypermethylation. *Int J Cancer* 80:439–443.
137. Lou W, Krill D, Dhir R, *et al.* (1999) Methylation of the CD44 metastasis suppressor gene in human prostate cancer. *Cancer Res* 59:2329–2331.
138. Kito H, Suzuki H, Ichikawa T, *et al.* (2001) Hypermethylation of the CD44 gene is associated with progression and metastasis of human prostate cancer. *Prostate* 49:110–115.
139. Woodson K, Hayes R, Wideroff L, Villaruz L, Tangrea J (2003) Hypermethylation of GSTP1, CD44, and E-cadherin genes in prostate cancer among US Blacks and Whites. *Prostate* 55:199–205.
140. Ekici S, Ayhan A, Kendi S, Ozen H (2002) Determination of prognosis in patients with prostate cancer treated with radical prostatectomy: prognostic value of CD44v6 score. *J Urol* 167:2037–2041.
141. Bergelson JM, Cunningham JA, Droguett G, *et al.* (1997) Isolation of a common receptor for Coxsackie B viruses and adenoviruses 2 and 5. *Science* 275:1320–1323.
142. Tomko RP, Xu R, Philipson L (1997) HCAR and MCAR: the human and mouse cellular receptors for subgroup C adenoviruses and group B coxsackieviruses. *Proc Natl Acad Sci USA* 94:3352–3356.

143. Okegawa T, Pong RC, Li Y, Bergelson JM, Sagalowsky AI, Hsieh JT (2001) The mechanism of the growth-inhibitory effect of coxsackie and adenovirus receptor (CAR) on human bladder cancer: a functional analysis of car protein structure. *Cancer Res* 61:6592–6600.
144. Li Y, Pong RC, Bergelson JM, *et al.* (1999) Loss of adenoviral receptor expression in human bladder cancer cells: a potential impact on the efficacy of gene therapy. *Cancer Res* 59:325–330.
145. Sachs MD, Rauen KA, Ramamurthy M, *et al.* (2002) Integrin alpha(v) and coxsackie adenovirus receptor expression in clinical bladder cancer. *Urology* 60:531–536.
146. Rauen KA, Sudilovsky D, Le JL, *et al.* (2002) Expression of the coxsackie adenovirus receptor in normal prostate and in primary and metastatic prostate carcinoma: potential relevance to gene therapy. *Cancer Res* 62:3812–3818.
147. Hemmi S, Geertsen R, Mezzacasa A, Peter I, Dummer R (1998) The presence of human coxsackievirus and adenovirus receptor is associated with efficient adenovirus-mediated transgene expression in human melanoma cell cultures. *Hum Gene Ther* 9:2363–2373.
148. Miller CR, Buchsbaum DJ, Reynolds PN, *et al.* (1998) Differential susceptibility of primary and established human glioma cells to adenovirus infection: targeting via the epidermal growth factor receptor achieves fiber receptor-independent gene transfer. *Cancer Res* 58:5738–5748.
149. Okegawa T, Li Y, Pong RC, Bergelson JM, Zhou J, Hsieh JT (2000) The dual impact of coxsackie and adenovirus receptor expression on human prostate cancer gene therapy. *Cancer Res* 60:5031–5036.
150. Lucas A, Kremer EJ, Hemmi S, Luis J, Vignon F, Lazennec G (2003) Comparative transductions of breast cancer cells by three DNA viruses. *Biochem Biophys Res Commun* 309:1011–1016.
151. Pong RC, Lai YJ, Chen H, *et al.* (2003) Epigenetic regulation of coxsackie and adenovirus receptor (CAR) gene promoter in urogenital cancer cells. *Cancer Res* 63:8680–8686.
152. Jarrard DF, Kinoshita H, Shi Y, *et al.* (1998) Methylation of the androgen receptor promoter CpG island is associated with loss of androgen receptor expression in prostate cancer cells. *Cancer Res* 58:5310–5314.
153. Kinoshita H, Shi Y, Sandefur C, *et al.* (2000) Methylation of the androgen receptor minimal promoter silences transcription in human prostate cancer. *Cancer Res* 60:3623–3630.
154. Sasaki M, Tanaka Y, Perinchery G, *et al.* (2002) Methylation and inactivation of estrogen, progesterone, and androgen receptors in prostate cancer. *J Natl Cancer Inst* 94:384–390.

155. Li LC, Chui R, Nakajima K, Oh BR, Au HC, Dahiya R (2000) Frequent methylation of estrogen receptor in prostate cancer: correlation with tumor progression. *Cancer Res* 60:702–706.
156. Freemantle SJ, Spinella MJ, Dmitrovsky E (2003) Retinoids in cancer therapy and chemoprevention: promise meets resistance. *Oncogene* 22:7305–7315.
157. Nakayama T, Watanabe M, Yamanaka M, *et al.* (2001) The role of epigenetic modifications in retinoic acid receptor beta2 gene expression in human prostate cancers. *Lab Invest* 81:1049–1057.
158. Maruyama R, Toyooka S, Toyooka KO, *et al.* (2002) Aberrant promoter methylation profile of prostate cancers and its relationship to clinicopathological features. *Clin Cancer Res* 8:514–519.
159. Yamanaka M, Watanabe M, Yamada Y, *et al.* (2003) Altered methylation of multiple genes in carcinogenesis of the prostate. *Int J Cancer* 106:382–387.
160. Viola MV, Fromowitz F, Oravez S, *et al.* (1986) Expression of ras oncogene p21 in prostate cancer. *N Engl J Med* 314:133–137.
161. Sumiya H, Masai M, Akimoto S, Yatani R, Shimazaki J (1990) Histochemical examination of expression of ras p21 protein and R 1881-binding protein in human prostatic cancers. *Eur J Cancer* 26:786–789.
162. Carter BS, Epstein JI, Isaacs WB (1990) ras gene mutations in human prostate cancer. *Cancer Res* 50:6830–6832.
163. Pergolizzi RG, Kreis W, Rottach C, Susin M, Broome JD (1993) Mutational status of codons 12 and 13 of the N- and K-ras genes in tissue and cell lines derived from primary and metastatic prostate carcinomas. *Cancer Invest* 11:25–32.
164. Gumerlock PH, Poonamallee UR, Meyers FJ, deVere White RW (1991) Activated ras alleles in human carcinoma of the prostate are rare. *Cancer Res* 51:1632–1637.
165. Burbee DG, Forgacs E, Zochbauer-Muller S, *et al.* (2001) Epigenetic inactivation of RASSF1A in lung and breast cancers and malignant phenotype suppression. *J Natl Cancer Inst* 93:691–699.
166. Dammann R, Li C, Yoon JH, Chin PL, Bates S, Pfeifer GP (2000) Epigenetic inactivation of a RAS association domain family protein from the lung tumour suppressor locus 3p21.3. *Nat Genet* 25:315–319.
167. Liu L, Yoon JH, Dammann R, Pfeifer GP (2002) Frequent hypermethylation of the RASSF1A gene in prostate cancer. *Oncogene* 21:6835–6840.
168. Wang Z, Tseng CP, Pong RC, *et al.* (2002) The mechanism of growth-inhibitory effect of DOC-2/DAB2 in prostate cancer. Characterization of a novel GTPase-activating protein associated with N-terminal domain of DOC-2/DAB2. *J Biol Chem* 277:12622–12631.

169. Chen H, Pong RC, Wang Z, Hsieh JT (2002) Differential regulation of the human gene DAB2IP in normal and malignant prostatic epithelia: cloning and characterization. *Genomics* 79:573–581.
170. Chen H, Toyooka S, Gazdar AF, Hsieh JT (2003) Epigenetic regulation of a novel tumor suppressor gene (hDAB2IP) in prostate cancer cell lines. *J Biol Chem* 278:3121–3130.
171. Tsihlias J, Kapusta L, Slingerland J (1999) The prognostic significance of altered cyclin-dependent kinase inhibitors in human cancer. *Annu Rev Med* 50:401–423.
172. Zhang P (1999) The cell cycle and development: redundant roles of cell cycle regulators. *Curr Opin Cell Biol* 11:655–662.
173. Padar A, Sathyanarayana UG, Suzuki M, *et al.* (2003) Inactivation of cyclin D2 gene in prostate cancers by aberrant promoter methylation. *Clin Cancer Res* 9:4730–4734.
174. Heiss NS, Gloeckner G, Bachner D, *et al.* (1997) Genomic structure of a novel LIM domain gene (ZNF185) in Xq28 and comparisons with the orthologous murine transcript. *Genomics* 43:329–338.
175. Dawid IB, Toyama R, Taira M (1995) LIM domain proteins. *CR Acad Sci III* 318:295–306.
176. Vanaja DK, Cheville JC, Iturria SJ, Young CY (2003) Transcriptional silencing of zinc finger protein 185 identified by expression profiling is associated with prostate cancer progression. *Cancer Res* 63:3877–3882.
177. Gitelman SE, Kirk M, Ye JQ, Filvaroff EH, Kahn AJ, Derynck R (1995) Vgr-1/BMP-6 induces osteoblastic differentiation of pluripotential mesenchymal cells. *Cell Growth Differ* 6:827–836.
178. Tamada H, Kitazawa R, Gohji K, Kitazawa S (2001) Epigenetic regulation of human bone morphogenetic protein 6 gene expression in prostate cancer. *J Bone Miner Res* 16:487–496.
179. De Pinieux G, Flam T, Zerbib M, *et al.* (2001) Bone sialoprotein, bone morphogenetic protein 6 and thymidine phosphorylase expression in localized human prostatic adenocarcinoma as predictors of clinical outcome: a clinicopathological and immunohistochemical study of 43 cases. *J Urol* 166: 1924–1930.
180. Goessl C, Muller M, Heicappell R, *et al.* (2002) Methylation-specific PCR for detection of neoplastic DNA in biopsy washings. *J Pathol* 196: 331–334.
181. Kollermann J, Muller M, Goessl C, *et al.* (2003) Methylation-specific PCR for DNA-based detection of occult tumor cells in lymph nodes of prostate cancer patients. *Eur Urol* 44:533–538.

182. Cairns P, Esteller M, Herman JG, *et al.* (2001) Molecular detection of prostate cancer in urine by GSTP1 hypermethylation. *Clin Cancer Res* 7: 2727–2730.
183. Gonzalgo ML, Pavlovich CP, Lee SM, Nelson WG (2003) Prostate cancer detection by GSTP1 methylation analysis of postbiopsy urine specimens. *Clin Cancer Res* 9:2673–2677.
184. Goessl C, Krause H, Muller M, *et al.* (2000) Fluorescent methylation-specific polymerase chain reaction for DNA-based detection of prostate cancer in bodily fluids. *Cancer Res* 60:5941–5945.
185. Jeronimo C, Usadel H, Henrique R, *et al.* (2002) Quantitative GSTP1 hypermethylation in bodily fluids of patients with prostate cancer. *Urology* 60:1131–1135.